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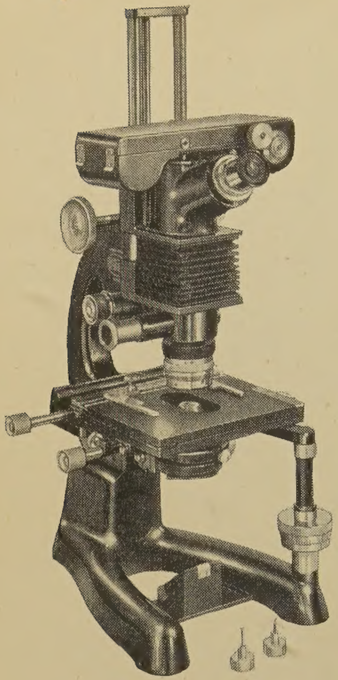

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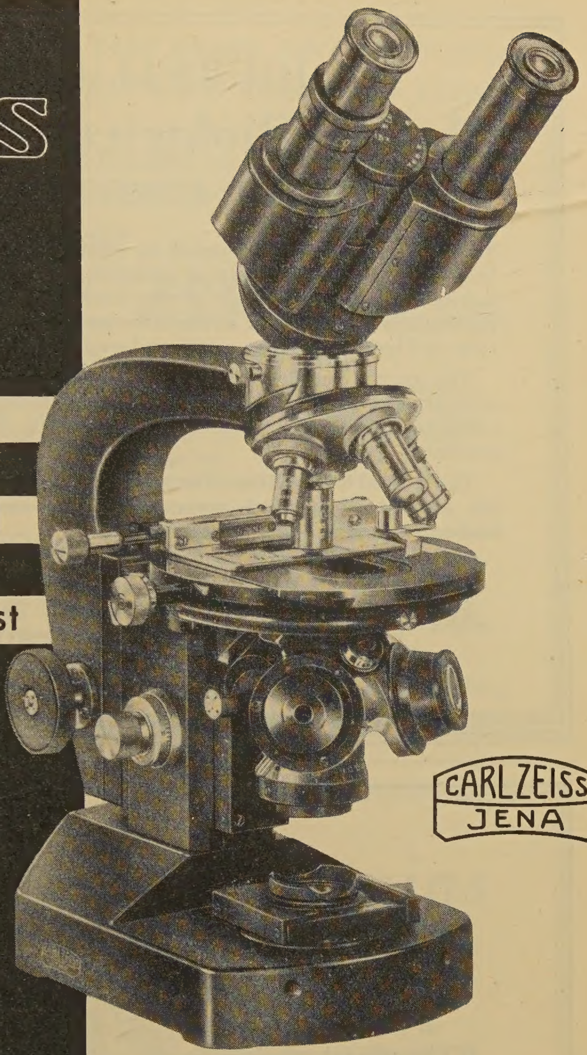

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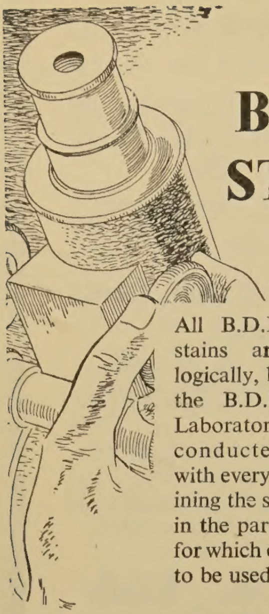

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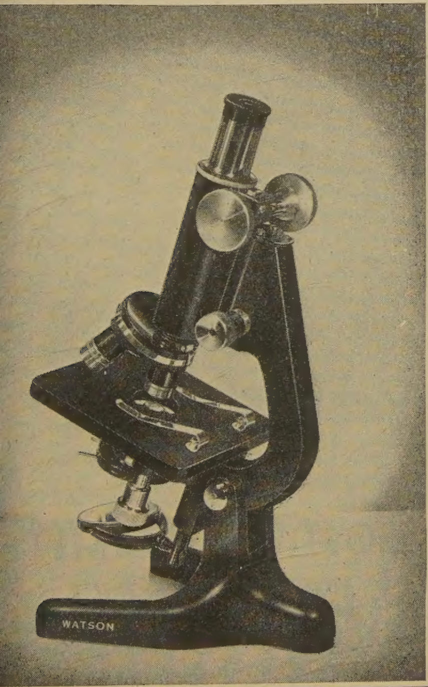

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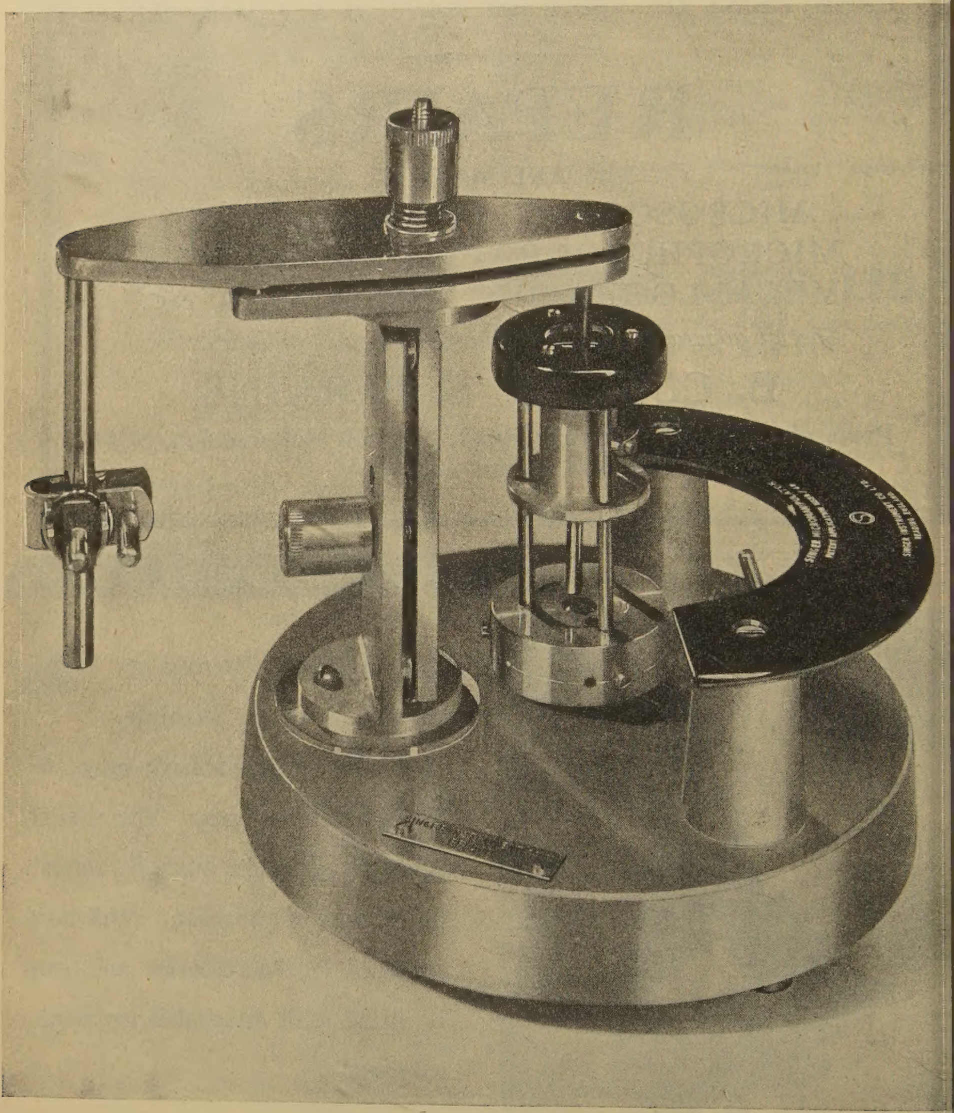

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The Tinctorial Behaviour of Human Mast Cells

By WILLIAM MONTAGNA, ARTHUR Z. EISEN, AND
ALLEN S. GOLDMAN

(*From Arnold Biological Laboratory, Brown University, Providence* 12, *Rhode Island, U.S.A.*)

With two plates (figs. 1 and 2)

SUMMARY

Mast cells in the skin differentiate from perivascular fibroblasts. The cells nearest the walls of the blood-vessels contain mostly sparse and small mast granules; in those farther removed from the blood-vessels the granules are more numerous and coarse. With weak solutions of toluidine blue, mast granules reveal maximal chromotropy at pH 5·0. At lower pH values not all of the granules stain; at higher ones the granules and the intergranular cytoplasm stain progressively more orthochromatically. After digestion with ribonuclease and staining with toluidine blue buffered to pH 4·0 or 5·0 the mast granules are cherry red and all traces of orthochromatic staining are abolished; when stained at pH 6·0 or above, however, the cytoplasm and the granules attain a strong blue stain as if they had not been digested in the enzyme. Preparations fixed in Helly's fluid may be washed in running water overnight and the mast granules show no diminution in chromotropy. The same sections may be stained, destained, and stained again at any desired pH with excellent results. Both the cytoplasm and the granules are Schiff-reactive, but the granules stain more intensely than the background. Sections stained with the periodic acid/Schiff technique and subsequently stained with toluidine blue reveal the mast granules brilliantly metachromatic, suggesting that the metachromatic and the Schiff-reactive substances, although co-existent, may be in fact separate elements. Mast granules, according to these tinctorial reactions, then, may contain 4 substances: (*a*) a protein cytoskeleton stainable with toluidine blue buffered to pH 6·0 or above; (*b*) some ribonucleic acid removable with ribonuclease and stainable with toluidine blue buffered to pH 5·0 or below; (*c*) an acid mucopolysaccharide which stains metachromatically; and (*d*) a Schiff-reactive substance.

THE observations in this paper were made on the skin of patients with *Urticaria pigmentosa* in which mast cells are very numerous. Biopsy specimens of skin stained under controlled conditions have made possible observations on the differentiation of mast cells and on the staining characteristics of the mast granules.

METHODS

Biopsy specimens of skin from six subjects with *Urticaria pigmentosa*, aged from 8 months to 57 years, were removed with a high-speed rotary punch and fixed immediately in chilled Helly's fluid. Five-micron paraffin sections were, (*a*) stained in 0·05 per cent. toluidine blue, the pH of which was adjusted from

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2·3 to 8·0 with McIlvaine's buffer (Montagna and others, 1951). (*b*) Comparable sections were treated with 0·1 per cent. ribonuclease adjusted to pH 6·5, at 36° C. for 2 hours, and then each slide was stained with toluidine blue buffered from pH 3·0 to 7·0. (*c*) Some sections were placed for 10 minutes in 0·05 per cent. periodic acid and 15 minutes in the sulphurous acid rinse used in the Schiff technique; they were then stained in toluidine blue as in steps *a* and *b*. (*d*) Some sections were washed 24 hours in running water before they were stained with buffered toluidine blue. (*e*) A series of sections from each specimen was treated with the method of McManus (1948), and one additional series, similarly treated, was also counterstained with toluidine blue buffered to pH 4·0 and 5·0.

OBSERVATIONS

In each of these specimens mast cells were numerous, polymorphic, and often bi- or multi-nucleated (fig. 1). In some specimens stained with toluidine blue the mast cells contain granules which are mostly coloured a dark lilac blue, whereas in others they are a reddish-purple. Many of the mast cells are in the form of fibroblasts which are arranged separately in concentric rings around small blood-vessels (fig. 1). The more centrifugal cells contain the coarsest and most chromotropic granules; the granules in the cells immediately around the blood-vessels stain orthochromatically with a faint metachromatic tinge.

With toluidine blue buffered to pH 2·3 most of the coarse granules stain metachromatically; the very small granules and some of the large ones stain a pale blue or remain unstained (cf. Montagna and Melaragno, 1953). At pH 3·0 and 4·0 there is an increase in the number of granules which stain metachromatically and at pH 5·0 all of the granules stain metachromatically. In addition to a variable complement of mast granules, each of the fibroblastic mast cells contains fine granules which stain orthochromatically. At pH 6·0 and above, the granules—those which stain orthochromatically as well as those which stain metachromatically—and the intergranular cytoplasm stain more intensely.

After incubation with ribonuclease and subsequent staining with toluidine blue buffered from pH 3·0 to 5·0 the mast granules appear red or reddish-purple, clearly outlined against the unstained background of the cytoplasm. None of the orthochromatic-staining granules seen in control sections are stained at these pH ranges. Above pH 5·0, however, some of the granules and the intergranular cytoplasm stain again orthochromatically. The metachromatic granules appear more blue than at lower pH values.

The sections treated with periodic acid and the sulphurous acid rinse before staining with toluidine blue show no tinctorial changes in the mast granules.

FIG. 1 (plate). A, fibroblastic mast cells radiating from a venule. One of the two endothelial cells shown on the left (arrow) has granules in the cytoplasm. Note binucleate cell (arrow). 675 ×.

B, enlarged detail of A to show the binucleate cell more clearly. 1425 ×.

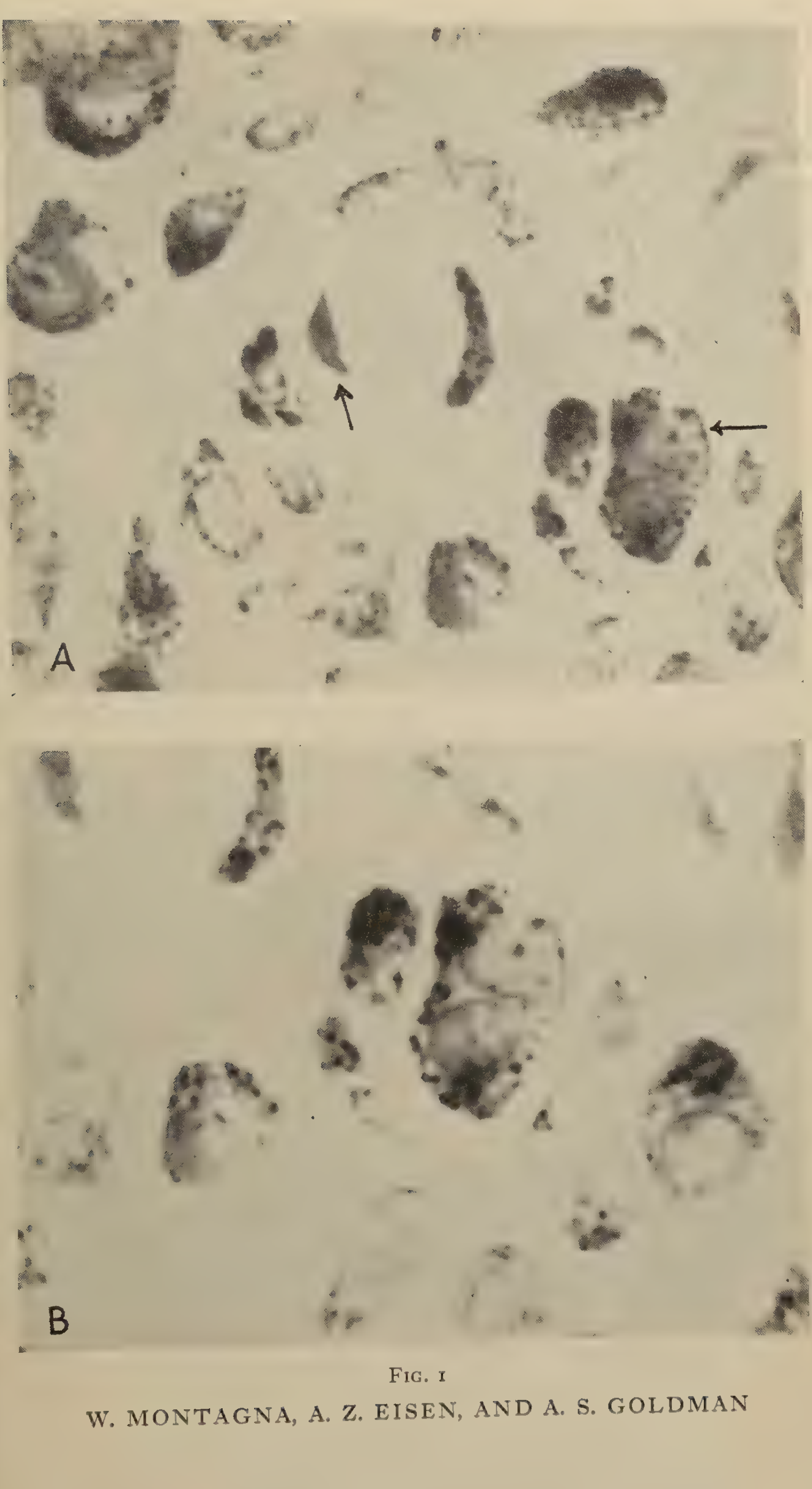

Fig. 1

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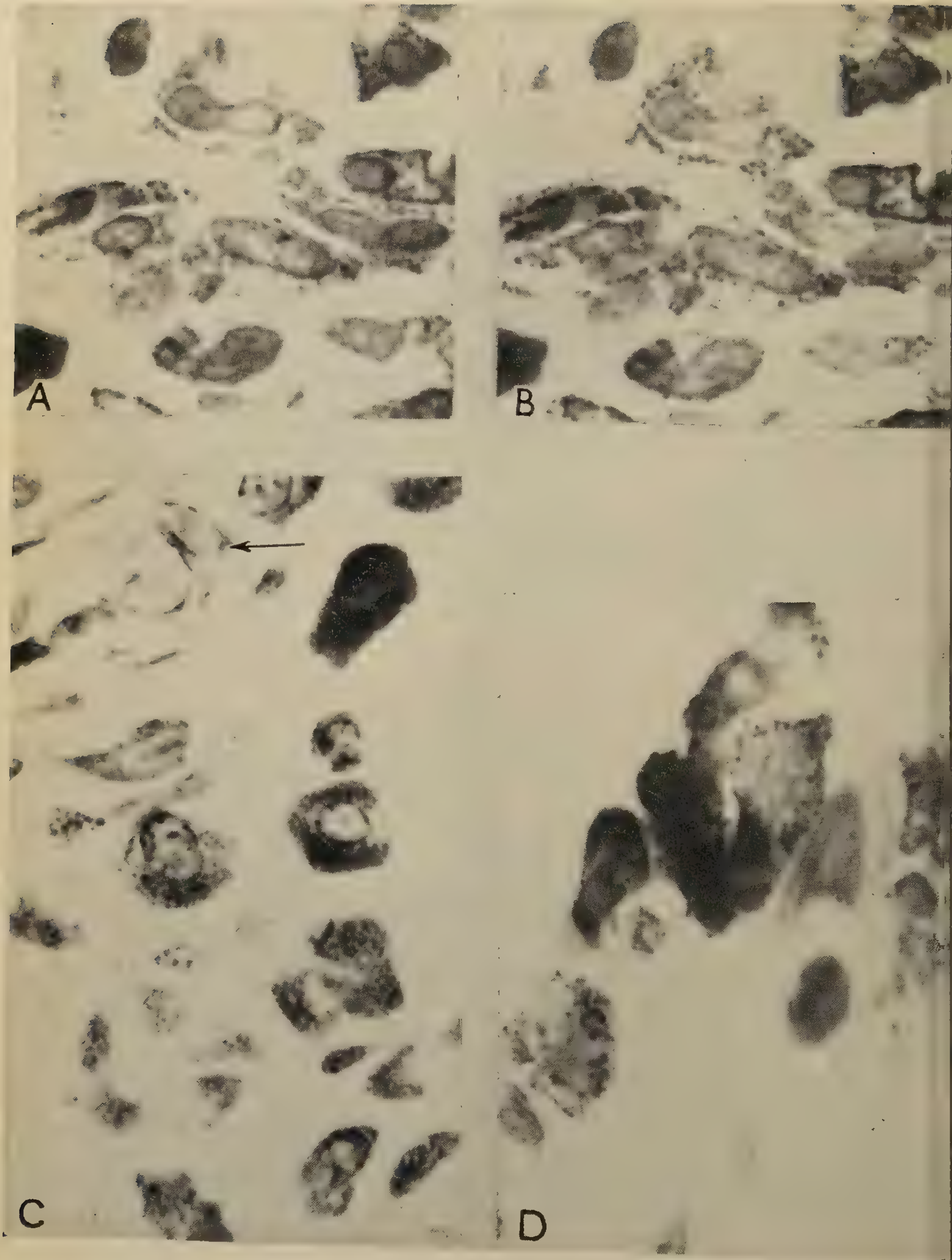

Fig. 2
W. MONTAGNA, A. Z. EISEN, AND A. S. GOLDMAN

Similarly, deparaffinized sections washed in running water for 24 hours show neither a diminution nor an alteration in the stainability of the mast granules. Sections can be stained with toluidine blue at any pH, photographed, destained with 80 per cent. ethanol, and stained again at any desired pH (fig. 2, A and B). This procedure can be repeated at will and the tissues behave each time as if they had not been stained before.

Mast cells are moderately Schiff-reactive. The granules stain more intensely than the intergranular cytoplasm but the granulation is poorly discerned. With the use of a combination of green and yellow Wratten filters in the path of the illuminating beam, the granules in every cell appear sharply demarcated (fig. 2, C). When sections coloured with the periodic acid/Schiff technique are then stained with toluidine blue buffered to pH 4·0 or above, the mast granules take up the stain so avidly that they appear essentially similar to those stained only with toluidine blue (fig. 2, D).

DISCUSSION

The majority of the mast cells in each of the specimens of *Urticaria pigmentosa* appears to be in a similar state of differentiation, recalling the observations by Oliver and others (1947) on mast cell tumours.

As in the connective tissue of the rat and the mouse (Bensley, 1952), mast cells are arranged in concentric circles around the walls of small blood-vessels and they appear to arise from perivascular cells which are indistinguishable from fibroblasts. Fawcett (1953) suggests that either the stem cells are located in the walls of the blood-vessels and then migrate into the surrounding connective tissue, or that the stimulus for the differentiation of mast cells radiates from the blood-vessels into the surrounding tissue. Mast cells in normal human skin are polymorphic and, with the exception of the metachromatic granules, they may be indistinguishable from fibroblasts (Montagna and Melaragno, 1953). It is difficult, however, to be sure that all of these are mast cells, since macrophages can phagocytize mast granules released by the cells (Fawcett, 1953).

In mast cells from the skin of man and of laboratory animals (cf. Friberg and others, 1951), the cytoplasm is never stained metachromatically, in contrast with the assertions of some authors (Glick and Sylvén, 1951; Julen and others, 1950; Sylvén, 1950, 1951). When stained with toluidine blue buffered to the same pH some granules stain a wine-red colour, others a purplish-blue (cf. Castiglioni, 1947). After incubation in ribonuclease the cytoplasm and those granules which in control sections stain orthochromatically at pH 3·0 to 5·0 no longer stain, but the mast granules show sharp metachromatic staining.

FIG. 2 (plate). A, field of mast cells stained with toluidine blue buffered to pH 4·0. 675 ×.

B, same field as A, destained and stained with toluidine blue at pH 5·0. 675 ×.

C, field of mast cells radiating from venule (arrow), all showing Schiff-reactive granules. Viewed through green and yellow Wratten filters. 675 ×.

D, mast cells first stained with the Schiff reagent and then with toluidine blue buffered to pH 4·0. The granules are strongly metachromatic. 675 ×.

When, however, enzyme-digested sections are stained with toluidine blue buffered to pH 6·0 or above, the mast granules re-acquire a blue cast and the intergranular cytoplasm stains again a light blue. Mast granules and the intergranular cytoplasm, then, may contain varying amounts of ribonucleic acid; the material stainable, after ribonuclease, with toluidine blue buffered to pH 6·0 or above, but not below, may represent the protein cytoskeleton.

In spite of the many cautions about the solubility of the metachromatic substances in water (Julen and others, 1950), sections deliberately left in running water for 24 hours or longer show no diminution in their ability to stain metachromatically.

Similar to the mast cells in animal tissues (Friberg and others, 1951; Glegg and others, 1952; Leblond, 1950), both the intergranular cytoplasm and the mast granules are Schiff-reactive and the cell appears homogeneously Schiff-reactive (Wislocki and Fawcett, 1951). The granules, however, are brought out sharply in every mast cell by using yellow and green light filters (fig. 2, c). Since the granules stain with toluidine blue even after they have been stained with the Schiff reagent (fig. 2, c), perhaps the Schiff reagent and toluidine blue stain two coexistent substances in the mast granules.

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Note on the Histochemical Localization of Glycogen and Pentosepolynucleotides in the Visual Cells of the Chick (*Gallus gallus*)

By M. RABINOVITCH, IVAN MOTA, AND S. YONEDA

(From the Laboratory of Cell Physiology, Department of Histology and Embryology, Faculty of Medicine, University of São Paulo, São Paulo, Brazil)

With one plate (fig. 1)

SUMMARY

1. Glycogen was studied in retinas of the chick (*Gallus gallus*), fixed in Gendre's fluid and stained by the periodic acid/Schiff method. In agreement with some of the earlier data on the subject, obtained with less suitable methods, glycogen was found in the paraboloid of accessory cones and in the characteristically shaped paraboloid of the rods.

2. Basiphilia, observable by means of the gallocyanin-chromalum stain and removable by ribonuclease or perchloric acid, was found in the inner segment of both chief and accessory cones, with the exception of the ellipsoid region.

INTRODUCTION

FEW data are to be found in the literature concerning the histochemical localization of glycogen in the visual cells of birds. The first reference to the subject seems to be that of Luna (1912), on *Columba livia*. This author described glycogen localization in the paraboloid of cells which seem to be accessory cones and rods in the sense used in the present paper. The methods used by Luna (Langhans and Vastarini-Cresi), however, are not specific (Lison, 1936), and no adequate controls were reported. Brammertz (1914), working on the same material, with Best's method after Carnoy's fixation, reported the presence of glycogen in the rod and cone layer, but gave no cytological details. Salivary digestion suggested that the staining was due to glycogen. Using absolute alcohol fixation and Best's carmine method, Schmitz-Moormann (1927) described the localization of glycogen in the myoid of cones of the pigeon's retina; rods did not stain. Uchiyama (1930), using the same method on the retina of the chick but apparently ignoring previous work on the subject, described staining of the paraboloid of the 'cones', rods being unstained. In a paper of which only an abstract could be obtained, Yoneyama (1932) observed in the same material the presence of glycogen in the ellipsoids of rods and cones. Fontana (1933), using the method of Fischer (considered

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to be unspecific by Lison (1936)) mentioned the presence of glycogen-containing granules in the visual cell layer of the retina of the pigeon and chick. Cytological details were not reported.

There is, therefore, no agreement in the literature as to the localization of glycogen in the rods and cones of the retina of birds. No reference could be found concerning the presence of pentosepolynucleotides in the visual cells of birds, although brief mention was made of their existence in the rod and cone layer of the retina of the rabbit by de Vicentiis (1949).

In this paper we present data on the localization of glycogen and pentosepolynucleotides in the visual cells of the retina of *Gallus gallus domesticus*, L., obtained by more adequate methods than those previously employed.

MATERIAL AND METHODS

Fifteen chicks of the Leghorn breed, 2–4 months old and weighing 200–400 gm. each, all light-adapted, were killed by decapitation. For the localization of glycogen the posterior poles of the eyes were immersed overnight at 4° C. in Gendre's (1937) fixative. The periodic acid/Schiff (P.A.S.) method according to Gomori (1952) was performed on 7μ paraffin sections, with occasional counterstaining with Harris's haematoxylin. Control sections were digested with saliva before staining. Pentosepolynucleotides were revealed by applying the gallocyanin-chromalum technique (Einarson, 1952) to paraffin sections of material fixed in Gendre's or Helly's fluid. Ribonuclease digestion was performed during one hour at 56° C. in a 0·1 mg./ml. crystalline ribonuclease solution in McIlvaine's citrate-phosphate buffer at pH 7·0. Control sections were incubated in the buffer alone. Extraction by perchloric acid was performed at 4° C. in a 3N aqueous solution for 12–16 hours. This higher concentration than that used by Erickson and others (1949) was necessary in order to remove all cytoplasmic basiphilic substance. Control sections were incubated in water in the same conditions. Slides stained with phosphotungstic acid haematoxylin were particularly favourable for the demonstration of the ellipsoids of the visual cells. The nomenclature of the visual cell organoids used in this paper is that employed by Walls (1942).

RESULTS

The structure of the visual cells in our material agrees with the description given by van Genderen Stort (1887) and Schmitz-Moormann (1927) for the pigeon, although simple cones could not be identified with certainty. Uchiyama's (1930) description of only one type of cone in the retina of the chick is therefore not confirmed.

Rods. These are easily recognizable in sections stained by phosphotungstic acid haematoxylin by their cylindrical and transversely striated outer segments. No negative image of either the oil globule or the paraboloid is found in our material. On the other hand, the periodic acid/Schiff reaction brings forth an intense staining of a localized region in the outer half of the inner segment of rods, which probably corresponds to the paraboloid (compare our fig. 1, A with Wall's figure 193*b* (1942, p. 660) of the rods of *Passer domesticus*). This region has frequently a conical shape, with its base pointing to the outer segment of the rods (fig. 1, A and B). Its height corresponds approximately to one-third of the height of the inner segment. This staining of the rod paraboloid does not occur after digestion by saliva (fig. 1, C). On the other hand, a distinct saliva-resisting P.A.S. reaction is found in the outer segment of the rods, in the form of alternatively stained and unstained disks (fig. 1, A, B, C). The positive reaction of the acromeres in mammal retinas to P.A.S. has been described by Day (1950) and by Lillie (1952), and was considered by the latter author as being probably due to a galactolipo-protein complex.

No staining of the rods could be observed by means of the gallocyanin-chromalum technique.

Cones. Both chief and accessory cones are found in our material (fig. 1, A). As was mentioned above, simple cones are not found in our sections. Chief cones do not stain with the P.A.S. method. Accessory cones show in the basal half of their outer segment a homogeneously stained, sharply limited, oval region (fig. 1, A, B), which we identify with the paraboloid. As this region does not stain in sections subjected to digestion by saliva (fig. 1, C), it is concluded that it contains glycogen.

Basiphilia removable by ribonuclease or perchloric acid is present in both the chief and the accessory cones in the inner segment, with the exception of the ellipsoid region (fig. 1, D, E, F). No staining of the outer segment is found. The paraboloid of accessory cones stains faintly and inconstantly (fig. 1, D, F). In oblique sections it can be clearly seen (fig. 1, F) that the distal part of the inner segment of the cones, up to but not including the ellipsoid, stains most intensely.

DISCUSSION

The localization of glycogen in the paraboloid of cones as here described confirms with more adequate methods the earlier data of Luna (1912) and Uchiyama (1930). The results of Schmitz-Moormann (1927) in the pigeon, and Yoneyama (1932) in the chick, pointing to the localization of glycogen respectively in the 'myoids' and 'ellipsoids' of visual cells are difficult to interpret. At least in the case of the former author the disagreement with our description is not due to difference in the nomenclature used, as Schmitz-Moormann recognizes the existence of a paraboloid in the visual cells as distinct from the myoid. The localization of glycogen in the paraboloid of rods possibly agrees with the data of Luna (1912) for the pigeon, but disagrees with those of Schmitz-Moormann (1927) on the same material and Uchiyama (1930) on the chick, as these authors did not find glycogen in the rods. The characteristic staining of the rod outer segments and the form and position of the rod paraboloids as visualized by the P.A.S. technique (fig. 1, A, B), suggest that it could be used in surveys of the distribution and numbers of rods in the bird retina.

No difference could be found by us in the glycogen distribution in the visual cells of 3 chicks that were dark-adapted for 4 hours and then killed under a faint red light when compared with 3 light-adapted chicks killed under daylight. It is interesting to observe that glycogen appears in the chick cones during the 18th day of incubation (Yoneyama, 1932), at a period when anaerobic glycolysis increases (Tamya, 1927), iodopsin can first be demonstrated (Bliss, 1946), and electric phenomena due to light-stimulation can be brought out (Hasama, 1941). This may suggest some relationship of glycogen to the function of the cones.

Our results pointing to the presence of pentosepolynucleotides in the cones of the chick retina should be checked by means of ultraviolet microspectrography. Nothing can be added at present on their physiological significance.

Thanks are due to Professor L. C. U. Junqueira for helpful suggestions. This study was aided by grants from the Comissão de Pesquiza Científica da Universidade de São Paulo and Conselho Nacional de Pesquizas.

FIG. 1 (plate). The photomicrographs represent sections of chick retinas after Gendre's fixation, stained by the periodic acid/Schiff (P.A.S.) technique with haematoxylin counterstaining (A, B, C), and by the gallocyanin-chromalum method (C, D, F). The scales represent 10μ.

A, note two double cones and one rod. Double cone 1 consists of a chief cone (above) and an accessory cone (below). Double cone 2 presents these cells in reverse order. In the chief cones can be seen the negative images of the oil globules and there is no staining by the P.A.S. method. The accessory cones present an ovoid glycogen-containing paraboloid. In the labelled cells there is some artificial retraction of the glycogen-containing substance. The labelled rod 3 and others that are found in the field, present a transversely striated outer segment, stained by P.A.S., and a conical glycogen-containing paraboloid in the inner segment.

B. Medium power view showing glycogen-containing paraboloids of accessory cones and of rods. The arrow points to one of the rod paraboloids, which are typically found in a more distal position than those of the accessory cones. The outer segments of the rods are stained by the P.A.S. method.

C. Section adjacent to the one shown in B, stained by the P.A.S. method after digestion with saliva (30 min. at 37° C.). The paraboloids of the rods and accessory cones no longer stain, but staining of rod outer segments is unaffected. Basiphilic regions are found distal to the unstained paraboloids (lower part of the photomicrograph).

D. High-power view showing cones stained by gallocyanin-chromalum. Observe the basiphilia of the inner segments of the chief and accessory cones. The arrow points to a double cone, with the chief component above and the accessory one below. Note the negative images of the oil globules and unstained ellipsoids. The section was incubated overnight in distilled water at 4° C.

E. Similar section extracted with 3N perchloric acid overnight at 4° C. before the gallocyanin-chromalum staining. The basiphilia of the inner segments of the cones no longer appears. Similar results were obtained with ribonuclease digestion.

F. Oblique section of a chick retina showing, diagonally, from the top left corner to the opposite one: nuclei of the rods and cones; paraboloids of accessory cones transversely sectioned and faintly stained; cytoplasm surrounding the paraboloids is moderately stained; stained chief cones (transversely cut) are also shown in this region. The next region belongs to a plane just proximal to the ellipsoids. The right-hand upper corner shows the region of the ellipsoids, oil globules, and outer segments.

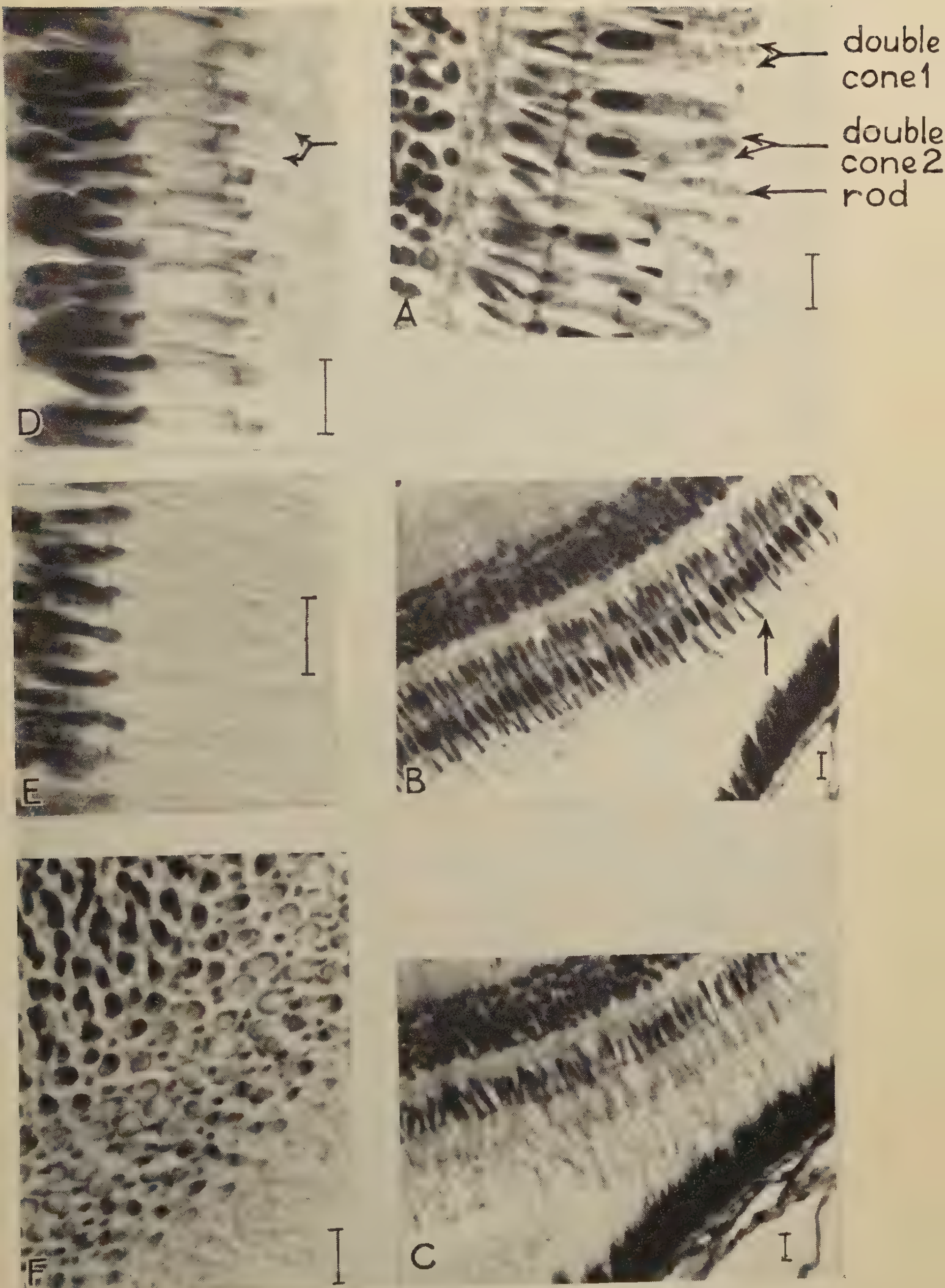

Fig. 1

M. RABINOVITCH, I. MOTA, AND S. YONEDA

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A Sharpening Machine for a New Type of Microtome Knife

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SUMMARY

A description is given of the manufacture of a new type of microtome knife made from selected portions of discarded low tungsten steel power hacksaw blades. A machine for sharpening this type of knife is described. Its main feature is a lapping plate of ¼ in. plate glass which can be adjusted for height to give cutting angles ranging from 24 to 32°, these angles being accurately reproducible on resharpening. Results of tests with a series of knives covering this range of cutting angles is given.

INTRODUCTION

ONE of the problems facing most workers wishing to cut thin sections is how to obtain a suitable cutting edge on a knife. Hillier and Gettner when describing their method for cutting thin sections suggested that the sharpness of the knife was one of the chief problems. Sharpening a standard microtome knife by honing on an oil stone depends entirely upon the skill of the operative; this is an art which is not easy to acquire. Although various methods of sharpening knives have been described in literature supplied by microtome manufacturers, they nearly all use some kind of hone 2 or 3 inches wide. Sharpening on such a stone necessitates drawing the knife backwards and forwards along the length of the stone, and at the same time taking it diagonally across the stone in order to cover the whole length of the blade. As this process is carried out by hand, pressure on the knife is variable, causing uneven sharpening along the cutting edge. Even if the knife edge is straight when leaving the makers it will not remain so for long under such conditions of sharpening, and sharpening will become more difficult each time it is honed.

An alternative method described in the booklet supplied with a Spencer Rotary Microtome model 820, makes use of the lapping principle. Here the knife is sharpened by lapping on a glass plate with suitable abrasive powders.

Having experienced the difficulty of sharpening a knife to a condition which is suitable for cutting fine sections of wool, the writers were led to investigate the possibilities of designing a less conventional type of knife. It was also considered that if a satisfactory knife could be designed a machine for sharpening it should be made. It was thought that by using a machine it should be possible to preserve the straightness of the edge and the cutting angle.

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THE KNIFE

Our first efforts were directed to making a knife the size of a safety razor blade, which could be mounted in the holder supplied with the Cambridge Rocking Microtome for fine sections. The knife was made from a portion, selected for straightness, of a tungsten steel power hacksaw blade. The selected piece of steel was rough-ground on the edges and then surface-ground to a thickness of $\frac{1}{16}$ in. A bevel angle of 12° was next ground towards one edge, care being taken not to draw the temper and also to preserve the flatness of the blade. The cutting edge was then produced by lapping with flour emery and oil on plate glass. The actual cutting angle was obtained by making a back as shown in fig. 1; in this instance the angle was 24°. The blade was pressed on to the lap by the finger tips and sharpened by giving it a rotary motion. Further lapping was then done with S.I.R.A. abrasive and oil and finally with jewellers' rouge and water; lapping being continued for a short time when the rouge was dry. The blade was not stropped after sharpening. Later when the machine was used for sharpening, Carborundum 2A700 WF paste was substituted for S.I.R.A. abrasive.

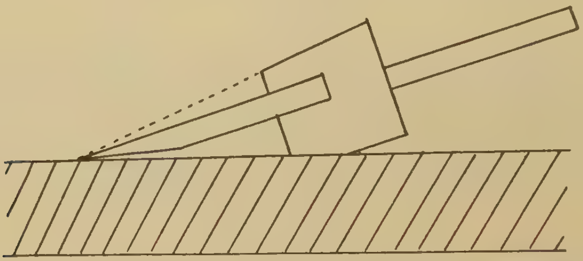

FIG. 1. Diagram of knife held in metal back as used for hand sharpening.

The useful cutting edge of our first knife was limited to 5 mm. by the design of the holder, but the results obtained with it led us to make others with cutting edges as long as that on a standard knife for the Cambridge Rocking Microtome. The same methods of manufacture were used as before.

THE SHARPENING MACHINE

This machine, fig. 2, was designed to perform automatically the motions of hand lapping, and only requires that the knife be turned over at intervals and adjustment made to the level of the lap to compensate for the bevel angle.

The machine consists of two units, a motor-driven gearbox which imparts the motion to the knife, and an adjustable stand for the lap, these units being mounted on a cast aluminium base-plate. The motor is a G.E.C. capacitor, 1/20th horse-power, giving 1,400 r.p.m. and is coupled to a gearbox worm-shaft driving through worm wheels with an 18 to 1 reduction, and two vertical shafts which project above the box and carry circular plates. Shoulder screws in tapped holes near the edges of these plates, and in line with the worm-shaft, drive a coupling bar, a triangular member being hinged on to this; at the apex of this triangle is a sleeve through which swivels a pin attached to the knife clamp. The hinge and swivel ensure that contact between the knife edge and the lapping plate is always maintained. Pressure on the knife during sharpening is only that exerted by its own weight and the weight of the clamp.

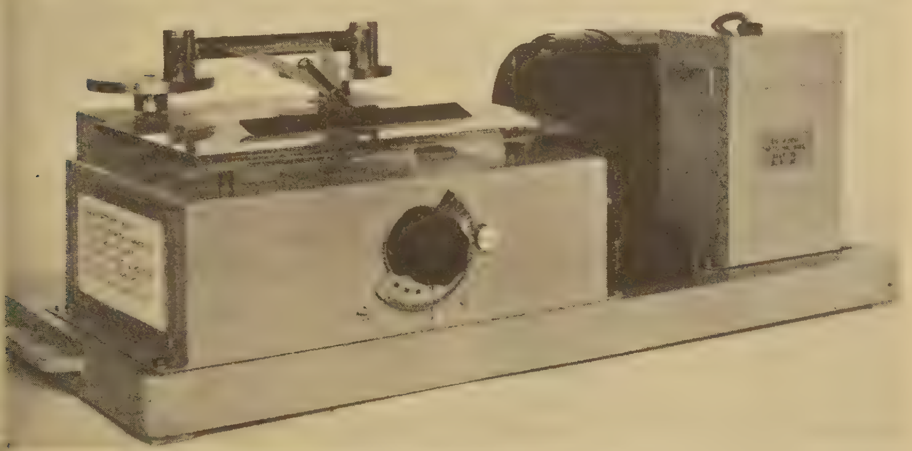

FIG. 2. View of the machine with the blade in position; control knob for setting the angle is shown on the front.

The plate glass lap rests on rubber pads, *a* in fig. 3, carried in cups fitted to the ends of four silver-steel pillars *b*. These are a smooth sliding fit in sleeves *c* fixed in the top plate of a frame which houses the adjusting mechanism. The weight of the glass plate resting on the pads is sufficient to keep the adjustable feet *d* on the lower ends of the pillars in contact with steel balls at the extremities of four levers *e*, positioned in pairs, and being free to rotate about two axles *f* mounted in the frame. The arms of these levers are in the ratio of 2 to 1, the balls on the ends of the shorter arms taking pressure from the undersides of two disks *g* fixed to the top of the stems which are free to slide in sleeves *h* fitted to the bottom plate of the frame. A horizontal shaft *i* with a control knob and dial *j* carries two eccentric cams *k* which operate on the top faces of the disks, transmitting movement of the control knob through the levers to the pillars. Attached to the control shaft is a serrated wheel *l* in which operates a spring-loaded detent pin *m*, serving to maintain the position of the movement at any setting. A movement of one tooth on the serrated wheel alters the cutting angle by $\frac{1}{2}°$.

Each knife we have made is marked with the settings of the control dial so that the cutting angle for each knife can be retained when it is resharpened.

Two straight-line graphs, one for each side of the blade, relating the angle to the graduations on the control dial, are used to give the correct settings for adjusting the level of the lap (fig. 4).

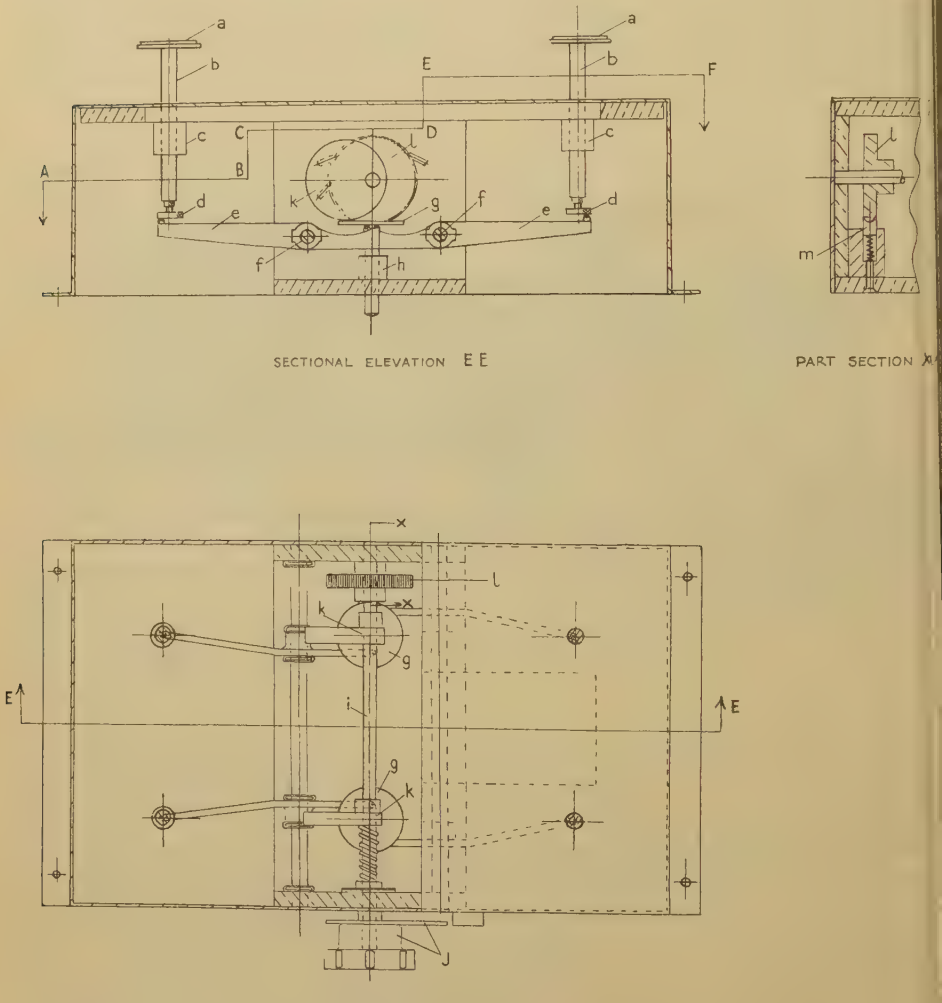

FIG. 3. Diagrams of the mechanism for adjusting the lap stand which controls the angle of sharpening.

RESULTS OF KNIFE TESTS

In order to test this type of knife a series of knives was made and sharpened to give a range of cutting angles. This range was as follows:

Knife A	24½°	Knife D	30°
,, B	26°	,, E	32°
,, C	28°		

During this test the clearance angle was kept approximately the same for ach knife whilst the cutting angle was altered by changing the knife.

Transverse sections of wool fibres were cut after coating the fibres with so-butyl methacrylate and then embedding in tropical-grade ester wax. All he knives were tried on the same block for cutting sections at a microtome eading of 1·0, 0·6, and 0·2 μ. The best results were obtained when knives with wide cutting angles were used. Knives D and E gave the best results; these were slightly better than the results from using knife C. Sections cut with knives A and B were poor; the edge on these acute-angled knives breaks down very rapidly; they are also more difficult to resharpen. The rapid breakdown of the edge is possibly the reason why the sections cut with these knives were so poor.

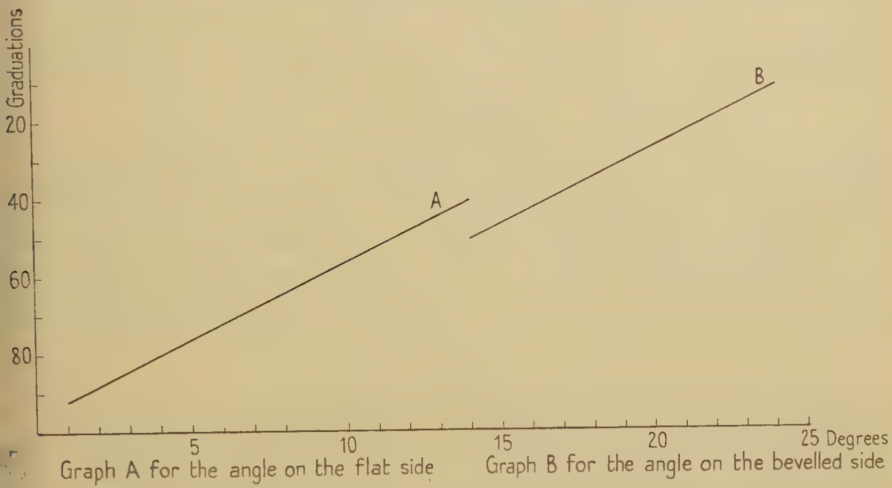

FIG. 4. Graphs used for determining the setting of the control knob to give the required cutting angle.

Similar results to the above were obtained when transverse sections of human hair were cut, but even with knives D and E the cuticle was torn away from some of the sections.

Other material has been cut with some of these knives, but a full range of cutting angles has not been tried on any other material except animal fibres. Knife B was used for vertical sections of the skin of the Herdwick sheep and of the frog, and transverse sections of a Guinea-fowl feather germ. There was some tearing of the connective tissue in 8 μ sections of Herdwick skin, but 8 μ sections of Guinea-fowl feather germ and 4 μ sections of frog skin were very good. Sections of deer skin, cut at 8 μ, and of Silky fowl feather germ, cut at 4 and 1 μ, were good when cut with knife D, although some of the 1 μ sections of feather germ did not stretch completely.

From these results we conclude that knives with wider cutting angles generally give better results than knives with more acute cutting angles. We have also found that such knives can be resharpened more quickly than those with acute angles.

We are grateful to Dr. A. B. D. Cassie and the Council of the Wool Industries Research Association for permission to publish this paper, and to Dr. A. B. Wildman, Chief Biologist, for his interest and encouragement in this work. We wish to thank Mr. G. Hines, of the workshop staff, for his skill and patience in making the machine and the knives; also our colleagues in the Biology department for supplying histological material, and particularly Mrs. C. M. Dymoke for her skill in sectioning the material.

The apparatus described in this paper is covered by Provisional British Patent No. 10660/53.

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The Effect of Phenylthiourea on Pigmentation by Melanin n the Developing Frog, *Eleutherodactylus martinicensis*

By NORMAN MILLOTT AND W. GARDNER LYNN

(*From the Department of Zoology, University College of the West Indies, Jamaica*)

With two plates (figs. 1 and 2)

SUMMARY

The black pigment cells in the skin, iris, and retina of *Eleutherodactylus martinicensis* (Tschudi) are melanophores. Under the influence of 0·01 per cent. phenylthiourea the melanin disappears in about 84 hours at laboratory temperatures. After administration of the substance for 5 days, discontinuance of treatment is followed by return of the pigment within 72 hours, to an extent at least equal to that normally attained n untreated controls. The stages of disappearance and reappearance of this pigment have been studied in the gross embryo, skin, iris, and retina, as well as in individual melanophores.

INTRODUCTION

THE effect of phenylthiourea on the colouring of animals by melanin has been known since the work of Richter and Clisby (1941). Up to the present, studies of this phenomenon have involved little or no histological evidence. One of us has previously shown that the administration of phenylthiourea to the developing embryos of the frog, *Eleutherodactylus*, causes a rapid and readily reversible depigmentation of the skin and retina (Lynn, 1948). In view of the importance of this in relation to the process of melanogenesis, it was decided to study in greater detail the time required to bring about disappearance and subsequent reappearance of melanin, and to correlate the gross changes already reported with changes in the melanophores.

MATERIALS AND METHODS

Eleutherodactylus martinicensis, like other members of the genus, has no aquatic tadpole stage, but lays large yolky eggs under loose boards or stones. The average number of eggs in a clutch is about twenty-four and the period between laying and hatching is approximately 3 weeks. The young hatch with the adult body form. The eggs used in this study were collected in the grounds of the University College, and were in the early neural groove stage. Each clutch of eggs constituted a separate experimental series, thus ensuring that all animals in a series were of precisely the same age.

Although the eggs of *Eleutherodactylus* are normally terrestrial, previous experiments have shown (Lynn, 1948) that the embryos are able to develop normally in water if the dense jelly coats which surround them are removed.

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It is thus possible to treat them with drugs, simply by keeping them in an appropriate solution.

The eggs were kept in the laboratory until the tail-bud stage was reached (stage 20, Lynn, 1942), and the jelly coats were then removed by dissection with fine watchmaker's forceps under a binocular microscope. The eggs in each clutch were divided into experimental and control groups, the controls being kept in tap-water, and the experimental embryos in a 0·01 per cent solution of phenylthiourea in tap-water. The two groups were kept side by side in the laboratory and the culture medium was changed daily. They were therefore subjected to the same environmental conditions, apart from the absence of phenylthiourea in the control series.

The pigment in the chromatophores of these embryos was identified provisionally as a melanin (see Millott, 1953) by tests based upon those formulated by Lison (1936).

In studying the disappearance of melanin, the embryos were frequently examined and some were fixed in Bouin's fluid at intervals of 12 hours. The experimental series for studying the reappearance of pigment after cessation of treatment consisted of embryos which had been kept in a 0·01 per cent solution of phenylthiourea for 5 days and then transferred to tap-water. Controls were maintained in tap-water from the beginning. Preliminary experiments showed that the return of melanin under these conditions was quite rapid and the animals utilized for histological study in these series were therefore fixed at 8-hour intervals. The fixed material was sectioned at 8 μ and stained lightly with acetic alum carmine, or in a few cases, with Mallory's triple stain.

OBSERVATIONS

The black pigment of the chromatophores

This pigment shows the characteristics of a melanin, the term being used in the unavoidably loose sense necessitated by lack of knowledge concerning the chemistry of these pigments. It occurs in the form of black granules, which are readily bleached by oxidizing agents such as hydrogen peroxide, bromine water, and 2 per cent. chromic acid. It is extremely resistant to solvents being insoluble in water, ethanol, benzene, acetone, pyridine, ether, petroleum ether, and chloroform, but differs from some melanins in being insoluble in ethylene chlorohydrin (see Lea, 1945). It also reduces ammoniacal silver nitrate directly.

The chromatophores may therefore be regarded as melanophores.

Gross changes in the pigmentation of the skin and retina

Direct observation of developing embryos shows clear and progressively greater differences in depth of pigmentation between experimentals and controls. This is shown in fig. 1, A–E. A comparison of A with E shows the increase in skin pigmentation, due to melanin, which occurs normally in 120 hours. A comparison of A with B to D, shows the progressive effect of phenylthiourea on skin pigmentation in the same area of similar embryos. The skin becomes lighter instead of darker, so that after 72 hours (B), individual melanophores were scarcely distinguishable, after 96 hours (C) they were indistinguishable, and after about 120 hours depigmentation of the skin was almost complete, revealing the iridophores in striking fashion (D). This condition contrasts markedly with that in the corresponding stage in the control series, shown at E, where the iridophores are almost completely masked by melanin. The evidence of the inhibitory effect of phenylthiourea on skin pigmentation is therefore unequivocal.

As reported already by Lynn (1948), the inhibitory effect was not confined to the skin, however, but extended to internal structures such as the melanin-bearing cells in the retina and around the brain. To observe this clearly it was necessary to examine sections of treated embryos, and to compare them with sections of the same areas of untreated embryos, as controls. Two such sections are shown in fig. 2, A being a section of the control, and B of the experimental embryo. The study of sections of embryos which had been subjected to phenylthiourea for progressively lengthening periods, reveals that the first signs of diminution in the amount of melanin appear after about 24 hours at laboratory temperatures. The loss of pigment appears more clearly at 36 hours, and was striking at 72 hours (compare fig. 2, A and B). After this the quantity of melanin in these areas was drastically reduced.

A similar series of sections can be used to demonstrate the reappearance of melanin when the treatment with phenylthiourea is discontinued. Photomicrographs of such a series of sections form the basis of fig. 2, C–F. After 120 hours' treatment, the melanin had almost completely disappeared from the sub-epidermal tissues of the head, iris, and retina. After treatment had been discontinued, this condition was maintained and no significant return of melanin could be observed, until between 32 and 40 hours. Its return became much clearer, however, in sections taken from embryos 56 hours after cessation of treatment (fig. 2, C and D), though the difference in the amount of melanin in the experimental embryo and the control is still marked. Between 56 and 72 hours, the return of melanin to the experimental embryos became so rapid, that not only did they attain parity in density of pigment, but some actually became darker than the corresponding controls (compare fig. 2, E and F).

Changes in the sub-epidermal melanophores

The normal melanophore. The structure of the normal sub-epidermal melanophore of *Eleutherodactylus* is illustrated in fig. 1, F. The general form is highly branched, the extent and disposition of the branches being very variable. It is possible that the precise form varies with factors in the external and internal environment, though no attempt has been made to study these. As would be expected, the most outstanding character is the abundant melanin in the cytoplasm, and when the pigment is dispersed, the cell processes become visible by virtue of this. The melanin exists in the form of separate round granules, measuring between 0·6 and 0·9 μ. These are so densely packed in some areas as to appear like a solid black mass. The remaining cytoplasm stains very faintly in acetic alum carmine. There is usually a single ovoid nucleus, but occasionally binucleate cells may be distinguished. In general, the nucleus is visible owing to its relative transparency as compared with that of the heavily laden cytoplasm, but its outline is usually obscured by the surrounding melanin, none of which was distinguished with certainty in the nucleus itself.

The effect of phenylthiourea on the melanophores. The effect of phenylthiourea on individual melanophores from the sub-epidermal tissue in the region of the eye, is shown in fig. 1, G, H, and I, taken respectively from embryos immersed in 0·01 per cent. phenylthiourea for 24, 36, and 72 hours, at laboratory temperatures. The progressive diminution in the amount of melanin is obvious. After 72 hours most of the pigment had disappeared, and by 84 hours the pigment was reduced to a few scattered granules.

Stage by stage, the differences between experimentals and controls appeared as follows. After 12 hours' immersion, no difference was evident; after 24 hours', a difference was observed in that the melanophores of the experimental series showed a decreased melanin content. This appeared as a diminution in the number of granules, rather than in their size, and as a result, certain areas of the cell often became clear of pigment. Usually the clear area appeared around the nucleus, which consequently became, for the first time, clearly defined in form and outline (fig. 1, G). At 36 hours the difference became marked and it was clear that in addition to the disappearance of more granules, many of those remaining were diminished in density, appearing brown instead of black. In the succeeding stages the effect steadily increased, until at 72 hours (fig. 1, I) only a fringe of pigment remained in the cell, and at 84 hours it became difficult to distinguish individual melanophores. For this reason no photograph was taken of this stage.

FIG. 1 (plate). A–E, surface views of the skin dorsal to the central nervous system of an embryo of *Eleutherodactylus martinicensis*. The embryos were photographed under the same conditions of lighting throughout. Owing to varying colour of the background, it was necessary to vary the exposures somewhat. The magnification is the same throughout.

A, from an embryo before treatment.

B, from the same embryo, after 72 hours in 0·01 per cent. phenylthiourea.

C, after 96 hours in the same solution. The white dots are iridophores exposed by the disappearance of melanin.

D, after 120 hours in the same solution.

E, the same region of the skin of an embryo of the same age as that photographed in D, but which had developed in tap-water. Note that the iridophores are obscured by a heavy deposit of melanin, a condition which markedly contrasts with that in the preceding photograph.

F–L, photographs of sub-epidermal melanophores. Fixed in Bouin's fluid; 8-μ sections stained with acetic alum carmine (with the exception of I, which was stained in Mallory's triple); the same magnification throughout. (Ilford filter No. 605.)

F, a normal melanophore from the sub-epidermal connective tissue in the eye region. Note the nucleus and nucleolus visible in the clear area toward the upper end of the cell.

G, H, and I, melanophores from the same region of embryos immersed in 0·01 per cent. phenylthiourea for 24, 36, and 72 hours respectively.

J, K, and L, melanophores from the same region of embryos which had been transferred to tap-water after 120 hours' immersion in 0·01 per cent. phenylthiourea for 8, 40, and 72 hours respectively.

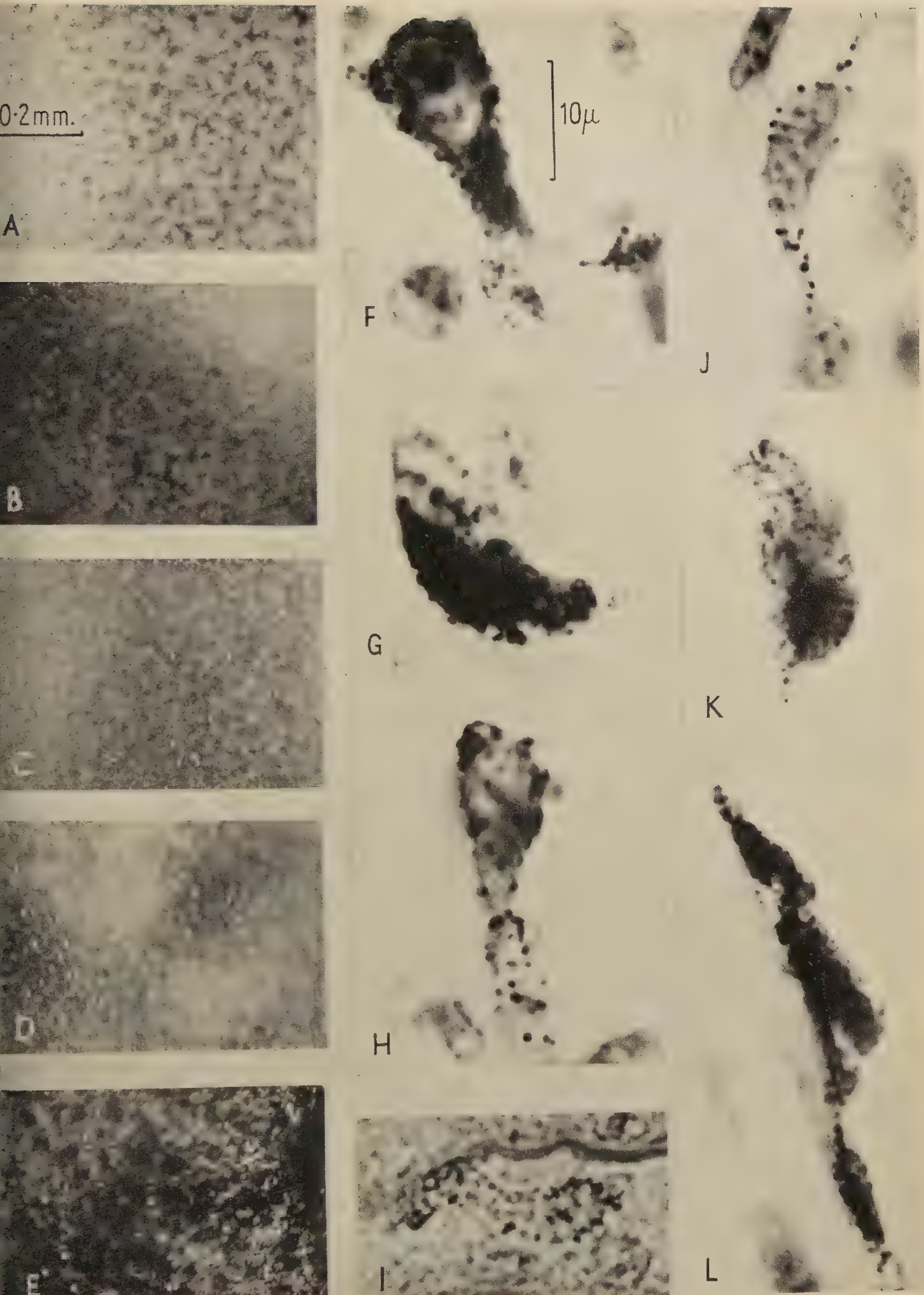

Fig. 1

N. MILLOTT AND W. G. LYNN

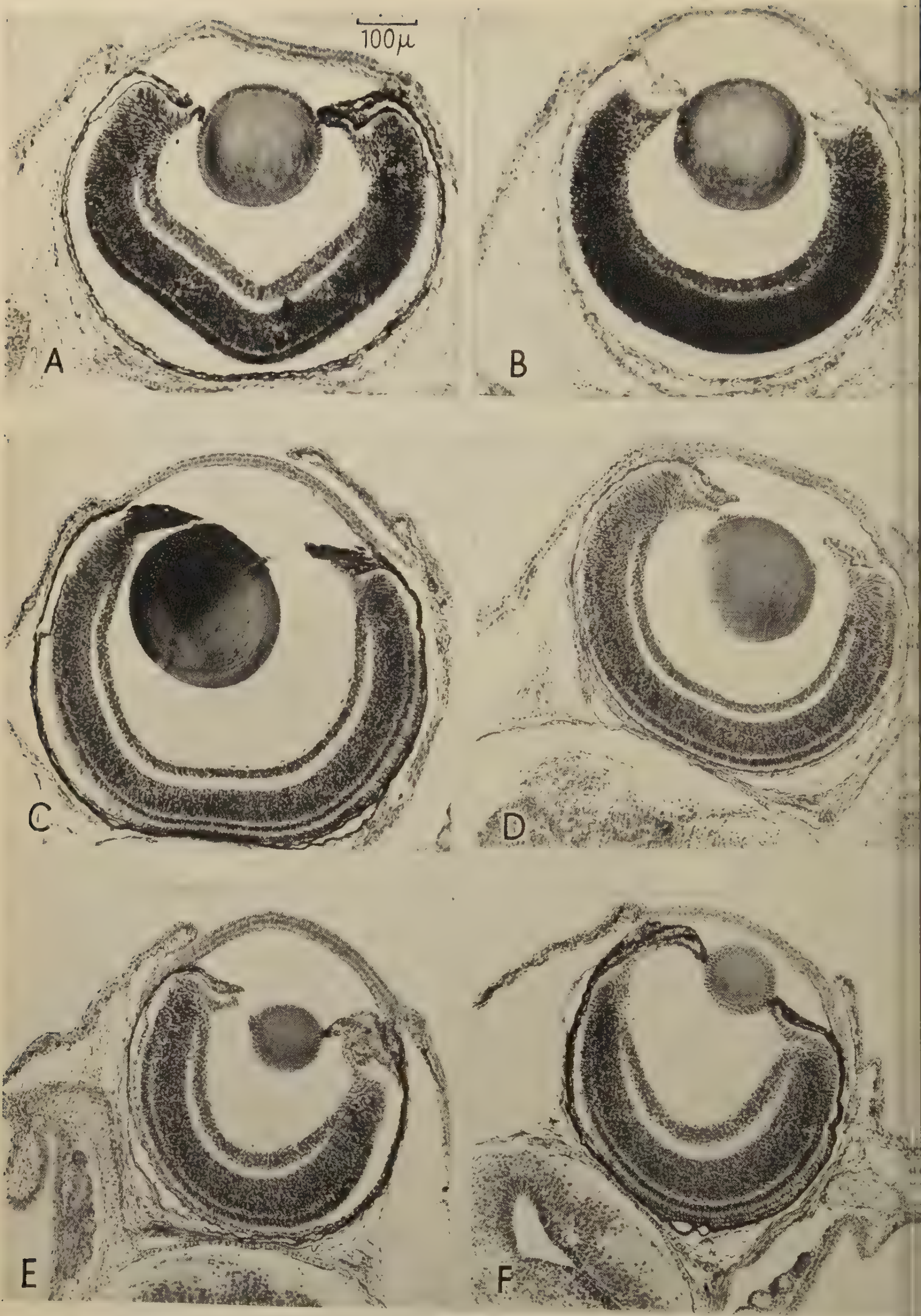

Fig. 2

N. MILLOTT AND W. G. LYNN

The return of the pigment is shown in fig. 1, J–L, taken from embryos that had been treated for 5 days with phenylthiourea and subsequently transferred to tap-water. Examination of sections of such embryos, fixed at 8-hour intervals, showed the first signs of melanin reappearing at 8 hours (J), scattered melanin granules defining the melanophore processes. At 40 hours the return of melanin appeared striking, for the number (though not the size) of the granules had greatly increased (K). By 56 hours the difference between the amount of melanin borne by the melanophores of the experimentals and their controls was greatly diminished, although the latter were still distinctly darker. It appeared that some of the melanin granules had become irregular and larger, but difficulties of observation, due in part to the density of the pigment, made it impossible to be sure on this point. After 72 hours the melanophores in the experimental embryos had regained their normal complement of pigment (L); indeed, in some cases the amount of pigment in the melanophores exceeded that in the controls, an observation which agrees with that already reported on page 19.

DISCUSSION

The inhibitory effect of phenylthiourea on melanin production has already been reported by authors such as Richter and Clisby (1941), Bernheim and Bernheim (1942), DuBois and Erway (1946), Dieke (1947), Lynn (1948), Lerner and Fitzpatrick (1950), and Lerner, Fitzpatrick, Calkins, and Summerson (1950). These observations involved both studies of living animals and chemical experiments, but no information concerning the action of phenylthiourea on individual melanophores has previously been presented.

Despite the uniformity in the findings of previous workers concerning the effect of phenylthiourea on pigmentation by melanin, its precise mode of action remains uncertain (see, for example, Richter and Clisby, 1941; Paschkis and others, 1944; Lerner and others, 1950). It has been generally assumed, however, that the substance acts upon the mechanism of melanin-formation,

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FIG. 2 (plate). Photomicrographs of sections of eyeballs of *Eleutherodactylus martinicensis*, showing the disappearance of melanin owing to the action of 0·01 per cent. phenylthiourea and the reappearance of the pigment when the action of the substance was discontinued. Fixed in Bouin's fluid; 8-μ sections stained in acetic alum carmine. (Ilford filter no. 605.) The magnification is the same throughout. In each case the experimental is shown on the right, alongside its corresponding control on the left.

A and B, control and experimental, showing the effect of phenylthiourea acting for 72 hours at laboratory temperature. In B the almost complete disappearance of melanin from the retina and iris can be seen. Since the sections are stained, the differences in melanin content do not appear so striking in the photographs as in the preparations, where it is clear that the retina and iris, as well as the sub-epidermal melanophores in B, are visible by virtue of the stain alone.

C–F show the return of the pigment after cessation of the action of phenylthiourea. The effect is seen clearly in the iris and retina. D, section from an embryo which had been returned to tap-water for 56 hours at laboratory temperature. F, section from an embryo which had been returned to tap-water for 72 hours at laboratory temperature. The greater density of pigment in the iris and retina of F, as compared with that in the same structures of the control, E, is noteworthy (see p. 19). C and E are the controls.

rather than upon the substance itself. If this is the case, then the results presented here show that the metabolic turnover of this pigment must be rapid.

The reversible effect of the substance, reported by us, agrees well with the observations of Richter and Clisby (1941) and Dieke (1947), who note the return of pigment to the skin of rats after the cessation of treatment. Also in harmony with the observations of Dieke is the fact that the repigmentation results, temporarily at least, in noticeably greater depth of colour in the experimental animals. In our experiments the speed with which the pigment returns on cessation of treatment with phenylthiourea is truly remarkable. It must be borne in mind, however, that embryonic cells have been studied exclusively here.

In the present experiments, treatment with 0·01 per cent. phenylthiourea caused complete depigmentation of the retina in 120 hours. In this respect, our results differ from those of Dieke (1947), who found that this substance, in doses sufficient to cause achromotrichia in rats, brought about no visible effect on retinal pigment. Though no attempt has been made here to determine whether the skin and the pigmented section of the retina are susceptible to different concentrations of phenylthiourea, it is noteworthy that Lynn (1948) found both equally affected by this substance in concentrations as low as 0·005 per cent.

It may be noted in conclusion that we have no evidence to support the idea that the depigmentation process is related to the well-known effects of phenylthiourea on the thyroid. Experiments carried out concurrently with the foregoing have shown that treatment with varying concentrations of thiourea brings about marked effects on the thyroid of these embryos, without discernibly affecting their pigmentation. This agrees with the findings of Richter and Clisby (1941), who showed that depigmentation was still produced by phenylthiourea in rats from which the thyroid was believed to have been completely removed.

We wish to acknowledge the valuable help of Mr. Hansard Taylor, technician in this Department, who prepared the sections used in this investigation. One of us, W. G. L., Professor of Biology, Catholic University of America, Washington, D.C., worked at the University College of the West Indies from September 1952 to September 1953 as a Fulbright Research Fellow.

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Histological and Polarization Studies of the Brush Border of the Proximal Convoluted Tubules of the Rat Kidney

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With one plate (fig. 1)

SUMMARY

The kidneys of rats were examined histologically after the intraperitoneal injection of haemoglobin and also after the intrarenal injection of a graphite suspension. During haemoglobin excretion spaces can be seen running across the brush border of the proximal convoluted tubules. These spaces are filled by the reabsorbed haemoglobin passing from the tubular lumen into the tubular cells. After intratubular injection of a graphite suspension, particles about 0·5 μ diameter cross the border and appear in the cell cytoplasm.

Examination with the polarizing microscope shows the brush border to have positive form and negative intrinsic birefringence with respect to the length of the striations.

These findings are considered in relation to the appearances found on electron microscopy. It is suggested that on present evidence it is most likely that the brush border consists of rodlets of the order of 300–600 Å diameter, with an orientated molecular structure at right angles to their length. These rodlets are gathered in tufts so that they diverge from one another. The spaces between the tufts at their bases is about 0·3–0·5 μ and at their tips they just touch one another.

MOST current textbooks of histology describe the brush border of the proximal convoluted tubules in a few lines. These brief descriptions vary somewhat, but most mention its striated appearance and compare it with the striated border of the small intestine. Von Mollendorff (1930) considered the matter in some detail. He suggested that it consists not of closely set rodlets as is commonly implied, but of holes with thin walls, resembling a honeycomb rather than a brush. He said that in longitudinal sections of the brush border he could, by focusing down, follow the walls of the holes through the thickness of the section. He also illustrated by a drawing a transverse section across the border showing a network of holes with thin, inter-connecting walls. Another view (Policard, 1944) is that the striations are produced by the passage of reabsorbed substances across the border.

It is well known that the mammalian proximal convoluted tubule can reabsorb haemoglobin (Rather, 1948) and certain other proteins (Oliver, 1948). Gérard (1935–6) has demonstrated that the open nephrons of amphibia can reabsorb much larger molecules and even minute particles of indian ink. Lison (1940) showed that the closed nephrons of the toad had this same

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property. The lining cells took up particles of indian ink after micro-injection directly into the tubular lumen. Lambert (1947) found that the cells lining cysts in a polycystic kidney could also take up indian ink.

In general these findings suggest that there are holes in the brush border. They might be spaces surrounding rodlets or holes as in a honeycomb.

I examined numerous stained sections, and although occasionally oblique sections of the brush border produced appearances somewhat resembling that illustrated by von Mollendorff, the structures involved are just at the limits of resolution and nothing absolutely unequivocal was ever seen. It is often very difficult to identify positively a brush border that has been cut transversely. In fig. 1, A, for example, the irregular masses in the lumen of the tubule show fine, closely set, pale areas producing a reticulated appearance resembling somewhat the 'honeycomb' of von Mollendorff. However, in this section the brush border is stained green whilst these masses are stained red, like the cytoplasm. The reticulated appearance is almost certainly due to fine vacuolation of cell cytoplasm.

The studies here described arose from the unsatisfactory results of the examination of ordinary stained sections. They consist of two parts. The first is a histological investigation of rat kidneys, after the intraperitoneal injection of haemoglobin or the intrarenal injection of a suspension of graphite particles. The second is a study of the polarization optics of the brush border.

HISTOLOGICAL STUDIES

The work of Baker (1951) suggested to me the use of haemoglobin in an attempt to demonstrate spaces in the brush border. He has demonstrated, very beautifully, spaces in the striated border of the small intestine. He showed that during fat absorption the spaces become filled with fat which can be readily seen in frozen sections stained with sudan black. As this border has many structural and histochemical resemblances to the brush border of the renal tubules, it was hoped that in a similar fashion spaces in the brush border in the kidney could be filled with haemoglobin during its reabsorption.

Experiment 1

Method. Albino rats weighing about 300 g. were injected intraperitoneally once on one day and twice the next with a solution containing about 800 mg. of frozen-dried human haemoglobin, i.e. a total of about 2·4 g. They were killed 16 hours after the last injection when they were still excreting large amounts of haemoglobin. The kidneys were fixed in Helly's fluid, dehydrated, and embedded in ester wax. Sections were cut at $2\,\mu$ and stained by a modified Mallory technique using acid fuchsin and fast green FCF.

Results. The structure of the kidneys of normal control rats is well demonstrated by this method. The brush border stains green, the cytoplasm and its granules red, and the basal granules of the brush border dark reddish-purple. The basal granules are quite prominent, and although they are described as basal a small amount of green staining brush border can be seen below them eparating them from the cell cytoplasm (fig. 1, B). Under low power objectives ey appear as a continuous line, but at higher magnification they can be esolved into distinct granules (fig. 1, A).

The appearances seen in the kidneys during the excretion and reabsorption f haemoglobin are very striking. Many of the proximal convoluted tubules re filled with haemoglobin which is stained bright red by the acid fuchsin. n the cytoplasm of many of the cells can be seen droplets of reabsorbed aemoglobin. The normal green-staining brush border is replaced by an lternation of green and bright red striations (fig. 1, C). The red colour is due o haemoglobin passing through and filling spaces in the brush border. In any of the tubules the haemoglobin-containing spaces do not appear to xtend right up to the edge of the border. They are separated from the aemoglobin in the tubular lumen by a narrow band of green-staining brush order (fig. 1, C and D). The spaces are about 0·3–0·5 μ in diameter in fixed nd stained sections.

From the size of the spaces it seemed possible that the cells of the rat ephron, like those of the closed nephrons of the toad, would be able to re-bsorb minute particles introduced directly into the tubular lumen. The econd experiment was undertaken in an attempt to demonstrate this.

Experiment 2

Method. Albino rats weighing about 300 g. were anaesthetized with ether. The left kidney was exposed through a loin incision. A suspension in water of graphite particles, 0·5–1·0 μ particle-diameter containing 0·39 per cent. graphite (kindly supplied by Acheson's Colloids Ltd., Plymouth), was injected into the cortex of the kidney with a very fine needle. About 0·2 ml. was slowly injected into the subcapsular cortex as the needle was being withdrawn. The rats were killed after 6 hours. The kidneys were fixed in formalin, dehydrated, and embedded in paraffin wax. Sections were cut and stained with Mayer's haemalum and eosin.

Results. The aim was to inject some of the graphite into the lumen of the tubules. This was achieved in one of three animals. Numerous particles could be seen in the lumen of a number of adjacent coils of proximal convoluted tubule. It was obviously the same tubule cut at different points along its length. In addition a number of particles had passed into the cell cytoplasm where they could be seen adjacent to cell nuclei and in the same plane of focus (fig. 1, E). These particles must have passed across the brush border, presumably in the spaces seen during haemoglobin reabsorption.

POLARIZATION MICROSCOPY

Schmidt (1943) found that the striated border of the small intestine of the tadpole shows positive form birefringence. It seemed likely in view of this and the reported findings on examining the brush border with the electron microscope that it too would show similar properties. An examination of the brush border with the polarizing microscope was therefore undertaken. It was only when this was partly completed that I became familiar with the work of Sjöstrand (1945–6) and Hillarp and Olivecrona (1946–7). With the knowledge of their findings the examination was extended. Our results are largely in agreement.

Method. Frozen sections of fresh unfixed rat kidneys were examined mounted in normal saline and in glycerol, refractive index 1·467. These sections proved very difficult to handle and the bulk of the observations were made on frozen sections of formalin-fixed kidneys. Frozen sections of formalin-fixed material were cut at 30μ. After being washed in water some were examined mounted in distilled water (R.I. 1·332), some in 50 per cent. human serum albumen (R.I. 1·405), some in propylene glycol (R.I. 1·432), and some in glycerol.

Other sections were dehydrated in alcohol and mounted in each of the following media: distilled water, tertiary butyl alcohol (R.I. 1·387), 50 per cent. serum albumen, chloroform (R.I. 1·446), glycerol, benzene (R.I. 1·500), oil of cloves (R.I. 1·534), methylene iodide 1 part+xylene 1 part (R.I. 1·606), methylene iodide 2 parts+xylene 1 part (R.I. 1·639), and pure methylene iodide (R.I. 1·75). The refractive indices were all measured with an Abbé refractometer except methylene iodide which has too high a refractive index for the instrument used.

Lastly an attempt was made to study the effect on the intrinsic birefringence of extracting the fat found in the brush border. Sections were placed in alcohol, in a saturated solution of sodium taurocholate in water, and in a 20 per cent. solution of Teepol (a proprietary anionic synthetic detergent of the alkyl sulphate type) for varying times. Similarly treated sections were examined by the polarizing microscope and stained for fat by the 3:4 benzpyrene

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FIG. 1 (plate). A, proximal convoluted tubule showing brush border with well-defined basal granules. The faintly reticulated material in the lumen in the upper part of the field is cytoplasm from cells of the other wall of the tubule. Acid fuchsin and fast green FCF. Ilford microfilters 3 and 4.

B, proximal convoluted tubule showing brush border with basal granules appearing as a line and separated from the cell by a narrow band of brush border. Acid fuchsin and fast green FCF. Ilford microfilters 3 and 4.

C, proximal convoluted tubule during reabsorption of haemoglobin. The haemoglobin which fills the tubular lumen is also filling spaces in the brush border as it passes across it into the cells. Acid fuchsin and fast green FCF. Ilford microfilters 3 and 4.

D, proximal convoluted tubule during haemoglobin reabsorption. In this, as in the previous figure, the haemoglobin-filled spaces are separated from the lumen at some points by a narrow band of apparently intact brush border. Acid fuchsin and fast green FCF. Ilford microfilters 3 and 4.

E, longitudinal section of tubule showing particles of graphite in the lumen. In the cells on the left near the two adjacent nuclei can be seen a number of fine intracellular particles. Haemalum and eosin. Ilford microfilters 3 and 4.

F, unstained frozen section under crossed polaroid screens with full compensation in one axis. The brush border shows up brightly in the axis at right angles.

G, proximal convoluted tubule photographed in non-polarized light. In this section the lumen is lined by brush border sectioned along its long axis (in polarized light this shows marked birefringence) and filled by the brush border of the other wall of the tubule cut transversely (this shows no birefringence). Acid fuchsin and fast green FCF.

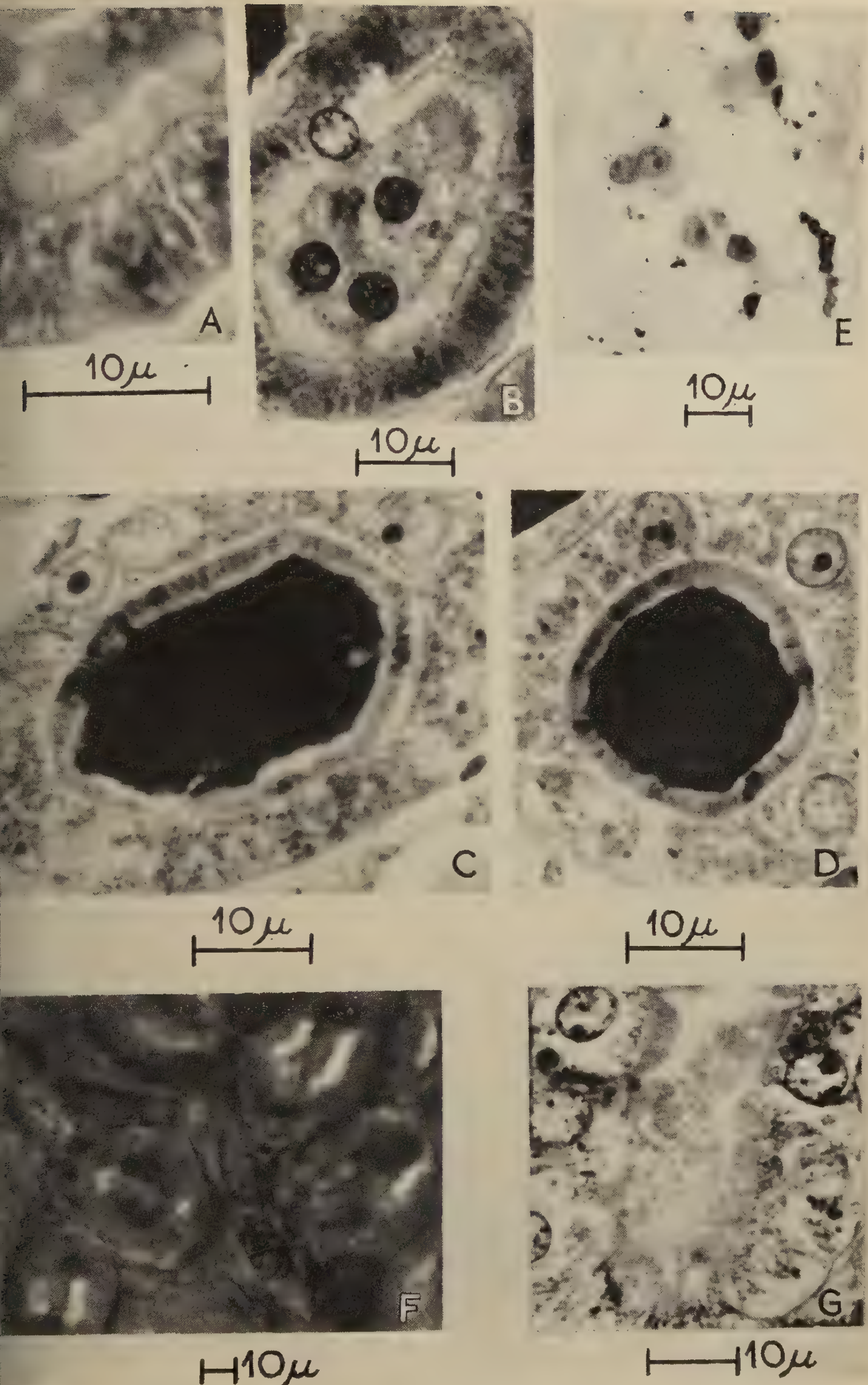

Fig. 1

D. B. BREWER

ethod of Berg (1951) and the propylene glycol sudan IV method (Pearse, 53).

The polarizing microscope used was a Cooke, Troughton, and Sims ad- nced model polarizing microscope with a 1/30 λ rotating mica plate com- ensator.

Results. In fresh unfixed sections mounted in saline the brush border ows definite birefringence, positive in relation to the length of the striations. resh sections mounted in glycerol show a marked increase in birefringence nd a striking change in its sign. It becomes negative along the length of the riations. The brush border in formalin-fixed frozen sections shows the same irefringence and the same change on immersion in glycerol. Immersion in ropylene glycol and serum albumen also changes the sign of the birefrin- ence from positive to negative.

In dehydrated sections subsequently mounted in water the birefringence more marked than in undehydrated sections (fig. 1, F). It is again positive character. It can readily be seen in transverse and longitudinal sections of he proximal convoluted tubules and is more striking in the inner cortex than the subcapsular cortex.

The birefringence is readily detectable in butyl alcohol, slight but definite 50 per cent. serum albumen and chloroform. It cannot be detected in lycerol, benzene, or oil of cloves, but reappears in a mixture of equal parts f methylene iodide and xylol. It is still stronger in the mixture of 2 parts ethylene iodide and 1 part xylene, and in pure methylene iodide it is as trong as in distilled water.

Sections mounted in benzene were remounted in distilled water and the irefringence reappeared. When present in dehydrated sections the bire- ringence was always positive. Owing to the marked variation in retardation of he brush border from tubule to tubule in any one section, measurements of etardation are not given.

It is seen that in undehydrated sections the birefringence undergoes a hange from positive to negative on immersion in media of high refractive ndex. This is an example of the so-called metatropic reaction (Bear and chmitt, 1936–7). The birefringence of dehydrated sections shows the typical orm birefringence curve on immersion in fluids of a wide range of refractive ndices.

Sjöstrand (1945–6) found that the brush border shows form birefringence. illarp and Olivecrona (1946–7) confirmed this and also described the meta- ropic reaction of undehydrated sections, although they found that sections ounted in water were isotropic. The reaction they suggested was due to a ombination of form and intrinsic birefringence, the intrinsic birefringence eing due to orientated lipide.

Fat can be stained in the brush border both by the propylene glycol sudan V technique and by the benzpyrene method. Five minutes' immersion in lcohol is sufficient to abolish almost completely the staining reaction for at. The negative birefringence disappears after only a few seconds in alcohol.

Such sections show an undiminished staining for fat. Immersion in bil salt solution for up to 2 hours was without effect either on the staining reactio for fat or on the intrinsic birefringence. The 20 per cent. solution of Teep abolished both the staining reaction for fat and the intrinsic birefringence i 5 minutes.

Besides the smooth muscle of the blood-vessels and the perivascula collagen, the tubular basement membrane, the basement membrane o Bowman's capsule and of the glomeruli all show birefringence. In all it i positive circumferentially in transverse section and positive along the lon axis of the wall in longitudinal sections. It is reduced in benzene and oil o cloves but it never completely disappears, so that it consists of both intrinsi and form birefringence.

DISCUSSION

The results of the histological part of the investigation show that in the ra there are spaces in the brush border of the proximal convoluted tubules tha become filled with haemoglobin during its reabsorption. In whichever plan the individual tubule is cut the spaces always appear to be about 0·3–0·5 wide. Between the spaces are apparently solid portions of brush border o about the same width. When particles of graphite are introduced into th tubular lumen they can pass across the border into the cell cytoplasm.

Studies with the polarizing microscope yield some information about th possible nature of the walls between the spaces. The metatropic reaction, th change in sign of the birefringence on mounting undehydrated sections i media of high refractive index, is generally taken to indicate the presence o positive form and negative intrinsic birefringence (Mitchison, 1952). I water the positive form birefringence predominates. Media of higher refractive index reduce the form but do not affect the intrinsic birefringence and s the total effect is to change the sign from positive to negative. The combination of positive form with negative intrinsic birefringence indicates tha the micellar axes are parallel to the striations of the border and the molecula axes at right angles.

The simplest explanation of the form birefringence is that the walls ar made up of rodlets of a diameter small in relation to the wavelength of ligh (i.e. of the order of 500 Å).

Such an interpretation was put forward by Schmidt (1943) to explain his findings in the striated border of the small intestine. Flagella, cilia, and sperm tails also show positive form birefringence. Schmitt, Hall, and Jakus (1943 showed by electron microscopy that the basis for this in all these structures is the presence of long fibrils 300–600 Å wide running along their length.

However, there is another possible explanation of these findings. As was pointed out by Schmitt (1944), the finding of positive form birefringence need not mean that the submicroscopic particles are rodlets. In the case of the brush border all that is required is that the long axes of the particles are parallel to the visible striations. They could then be either rodlets or platelets.

owever, platelets so arranged would appear anisotropic in one axis, e.g. in ansverse section of the tubule, and isotropic in a direction at right angles. his is not so, but if it is assumed that the platelets are arranged around pores e parts of the platelets viewed tangentially would show positive form bire-ringence along the striations of the border, whilst those running across the ptical axis of the microscope would be isotropic. Such an arrangement ould show positive form birefringence in all axes at right angles to the triations. A similar effect, on a larger scale, is produced by the concentric rotein layers of the myelinated axon sheath (Bear and Schmitt, 1936–7; hin and Schmitt, 1936–7).

Theoretically it is possible to distinguish between these two possibilities by xamining the brush border cut in transverse section. If it consists of rodlets t should appear isotropic, but if of lamellae arranged around pores each pore vall will be positively birefringent circumferentially as are the basement nembrane of the tubules and the protein layers of the axon sheath. It is ather difficult to examine the brush border in transverse section. One has to e certain that it is in fact brush border and that it does not include cell ytoplasm. The section would have to be truly transverse, as oblique sections f rods would show some birefringence. Further, such sections of brush order might be too thin for their birefringence to be readily detectable.

An attempt was made to examine the brush border in transverse section. araffin sections cut at 2μ and stained with acid fuchsin and fast green FCF vere searched for suitable fields. When one was found (fig. 1, G), the cover lip was removed and the section taken down to water. Examination with the olarizing microscope shows marked birefringence in most of the brush orders. In the tubule showing the brush border cut transversely and longitudinally, that cut longitudinally was strongly birefringent, while that cut ransversely filling the lumen showed no detectable birefringence with the 1/30 λ compensator. In making this observation it was unfortunately necessary to use paraffin-embedded material and to stain the sections so that the brush border could be identified. Nevertheless, the brush borders cut longitudinally still showed strong birefringence in sections cut at 2μ. If it consists of concentric lamellae about pores one would have expected it to still show birefringence in transverse section. Despite these unavoidable difficulties this observation is evidence in favour of the view that the brush border consists of rodlets. Sjöstrand (1945–6) also examined the brush border in transverse section and found it isotropic.

The nature of the probable molecular arrangement underlying the intrinsic birefringence is more doubtful. Until recently this combination of negative intrinsic with positive form birefringence in which the negative birefringence is readily abolished by exposure to alcohol had been interpreted as being due to an orientated micellar structure of protein with lipide molecules arranged at right angles, e.g. in the crustacean nerve axon sheath (Bear and Schmitt, 1936–7) and in the brush border (Hillarp and Olivecrona, 1946–7). However, this interpretation has recently been criticized (Mitchison, 1952). It depends on the demonstration of lipide in the structure concerned and on the assumption that exposure to alcohol only removes the lipide and leaves the protei micelles unaltered. As was pointed out by Mitchison, alcohol produces i effect very rapidly. In fact it was not found possible to remove sections fro alcohol sufficiently quickly to leave unchanged the intrinsic birefringenc Because of this very rapid action of alcohol he suggests that it might produc its effect by denaturing or dehydrating the protein molecules. He propose a structure of protein micelles made up of molecules folded like Chines crackers so that the axes of molecules are at right angles to those of th micelles. Alternatively, he suggests the protein might be in piles of disks wit the molecular chains parallel to the disk faces or in spirals. Alcohol could the abolish the intrinsic birefringence by disorganizing the alignment of th protein chains.

In the brush border the lipide is very readily removed both by alcohol an Teepol and at the same time the intrinsic birefringence disappears. However alcohol of course dehydrates the protein structure and Teepol is likely t denature it (Putnam, 1948). The fact that the intrinsic birefringence disappear before the fat is extracted by alcohol might be due to the disarrangemen of orientated lipide molecules. These findings then can be explained by th assumption of an orientated molecular structure of either lipide or protei and do not provide conclusive evidence in favour of either.

The reported studies of the brush border with the electron microscop have produced varying ideas as to its finer structure. Gautier and Bernhar (1950) say that the border is made up of complex branches and lamellae tha join with one another to form very irregular pores quite unlike the ver regular structures seen during haemoglobin reabsorption. In transvers sections they illustrate irregular pores surrounded by fine inter-connectin walls. The findings reported in two studies from America are quite different Pease and Baker (1950) found a regular structure of fine rodlets which the illustrate very convincingly both in longitudinal and transverse section. Thes fine processes are only about 300 Å diameter and the spaces between them ar about the same size. The findings of Dalton and others (1950–1) are similar They also found rodlets, but they appeared to be gathered in little tufts each made up of numerous processes. In each tuft the rodlets diverge from on another so that the space between the tufts is gradually reduced. At the bas there are spaces about 0·5 μ between each tuft, but at their distal ends th diverging processes almost touch.

In the most recent paper on this subject by Sjöstrand and Rhodin (1953 the illustrations are technically of the highest standard yet published. They also found the brush border to be made up of rodlets. They described them as ducts and in their illustrations they have a thin dense wall and a lumen filled with a less dense substance. They are closed at each end by the wall s the description 'duct' is rather a misnomer. When cut transversely they appea circular in cross-section. According to Sjöstrand and Rhodin the surface o the brush border adjoining the lumen is smooth and covered by a continuou embrane. This is difficult to make out from their illustrations as none of em includes identifiable tubular lumen and this is contrary to the finding at the tubular cells can take up particles of graphite.

Pease and Baker, Dalton and others, and Sjöstrand and Rhodin all agree in nding minute closely set rodlets in the brush border. They disagree regarding their dimensions. Dalton and others find them to be 700 Å in diameter, jöstrand and Rhodin 600 Å, and Pease and Baker 300 Å. However, any of hese findings would produce form birefringence, but only Dalton and his olleagues found a structure of rodlets arranged in tufts consistent with the inding of spaces in the brush border during haemoglobin reabsorption.

From evidence at present available it seems most likely that the brush order consists of a complex arrangement of rodlets roughly 300–600 Å in iameter. At right angles to the long axis of the rodlets is an arrangement of arallel-orientated molecules of either lipide or protein. These rodlets are set in ufts each possibly arising in a basal granule. Between the tufts are spaces bout 0·5 μ diameter, along which the reabsorbed glomerular filtrate passes nd along which particles of graphite of about this diameter could also pass. The arrangement of rodlets in diverging tufts might account for the fact that n many tubules the haemoglobin-filled spaces are separated from the lumen y a narrow, apparently intact band of brush border.

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The Orientation of the Optic Axis of Spicules of *Leucosolenia complicata*

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Summary

The orientation of the optic axes of the spicules of *Leucosolenia complicata* has been udied by using isolated spicules and longitudinal sections of the oscular tubes. For i- and quadri-radiates the mean angle of inclination of the optic axis to the tube all varies with the distance of the spicule from the oscular edge, particularly over the rst 200 μ. The spicule form likewise varies with position, but is not closely correted with the orientation of the optic axis: two spicules can have the same optic angle ut different shapes, or the same shape and yet different optic angles. For curved onaxons the optic axis is directed almost at right angles to the spicule axis at the istal end, and the spicule curvature is independent of the position in the tube. lender monaxons, on the other hand, have an optic axis coincident with the spicule ngth. It seems a general rule, at least for spicules of *L. complicata* and *L. coriacea*, hat the optic axis of the first formed calcite rudiment lies in the direction of the cell ivision (or cell association) that directly precedes spicule formation.

A mechanism is suggested for the orientation of the optic axis. This is explained in erms of an oriented overgrowth of the calcite upon an organic precursor, which is ructurally fixed and oriented within the formative cell complex. The arrangement of his complex, by mechanical factors arising during the development of the oscular ube, results in the orientation of the optic axis of the developing spicule. The hypohesis is in accordance with the observations of Minchin on spicule formation.

Contents

PAGE
NTRODUCTION 33
ETHODS 34
Determination of the direction of the optic axis 34
Sections 35
Isolated spicules 36
ESULTS 36
Appearance of spicules in surface-view 36
Optic angles of tri- and quadri-radiates 37
Curved monaxons 41
Slender monaxons 42
ISCUSSION 44
Mechanism of orientation of the optic axis 44
EFERENCES 47

Introduction

THE optical properties of calcareous spicules have been known for many years as a result of the work of Sollas (1885) and von Ebner (1887). These uthors independently observed that the spicules contain calcite, which gives hem birefringent properties revealed by the use of the polarizing microscope.

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The spicules in fact behave optically as though chiselled out from a sing homogeneous crystal, and von Ebner was able to classify the forms of spicul of different species in terms of the relations of the spicule rays to the princip crystallographic axes of this crystal. Each spicule thus possesses an optic ax (the trigonal axis of symmetry of the calcite crystal), which has been shown f species of *Sycon* to be oriented with respect to the sponge substance by vo Ebner (1887) and Bidder (1898). For example, the optic axis of the triradiat is nearly parallel to the axis of the radial tubes of *Sycon raphanus*, except their free conical ends, where it is nearly perpendicular to the surface (Bidder

The structure of the homocoelous ascon sponges is a great deal simpler tha that of the heterocoelous Calcarea because their oscular tubes are comparatively free of diverticula, and for this reason species of *Leucosolenia* have bee used by the author for the investigation of the mechanism of spicule orientation and the factors controlling spicule form, accounts of which will b published in due course. A study of the orientation of the optic axis of th spicules is an essential part of this research and the results are presented i this paper.

The spicules occurring in *Leucosolenia complicata* (Minchin) have alread been described by Minchin (1904), as seen when lying in isolation upon slide. There are four main types: quadriradiates, triradiates, curved monaxons, and slender monaxons. The triradiates are essentially similar to th quadriradiates but lack the fourth, or 'gastral', ray that projects into th spongocoel; the other three rays are embedded in the mesogloea of the tub wall and comprise a 'basal' ray, pointing to the base of the oscular tube, an paired, or 'oscular' (previously termed 'oral') rays diverging from the oscula end of the basal ray. The monaxons are single-rayed, some being stout, curve and fitted with lance-heads, others similar, but lacking the lance-heads, an the remainder, which are numerous, being straight, slender, and sharpl pointed. Fig. 7 summarizes the orientation of these spicules (see p. 43).

METHODS

Determination of the direction of the optic axis

When a spicule is mounted in balsam and rotated between crossed nicols the spicule alternately glows brilliantly and darkens completely four times in complete revolution of 360°, provided the optic axis of the calcite does no coincide with the microscope axis. The darkening occurs when the optic axi lies in the plane of vibration of the polarized light, or in the vertical plane a right angles to this, and the two planes are easily distinguishable by removing the analyser nicol: the spicule then appears faint when the optic axis lies in th first plane, but very distinct when in the plane at right angles. This arise from the greater difference between the refractive indices of calcite and balsam when only the ordinary ray (i.e. the ray that always vibrates at right angles to the optic axis) is being transmitted through the spicule. When the optic axis coincides with the microscope axis, these phenomena are not apparent, fo oth ordinary and extraordinary rays travel along the optic axis at the same elocity, and the refractive index of the calcite becomes that for the ordinary ay. In these circumstances the spicule remains invisible at all settings etween crossed nicols, and there is no change in distinctness on rotation, when the analyser nicol is withdrawn.

By such means it can be shown that the optic axis of the tri- and quadri-adiates of *L. complicata* lies in the plane containing the basal ray and bisecting he angle between the oscular rays, as shown by von Ebner and Sollas for other pecies. The angle between the optic axis and the basal ray can thus be determined by examining the spicule in side-view, when this plane is perpendicular o the line of vision, with a polarizing microscope. The angle taken, henceforth termed the 'optic angle', is defined for tri- and quadri-radiates as the maller angle of inclination of the optic axis to the line passing through the entral points at either end of the basal ray (fig. 5, A; see p. 41). This angle was measured to an accuracy of within $\pm\frac{1}{2}°$, three readings on either side of he appropriate plane of complete extinction of each spicule being made and he average taken.

Sections

The spicules can be examined in side-view and in position in the tube wall by cutting thick longitudinal sections that lie in or near an axial plane of the ube, i.e. a plane including the tube's central axis. Sections of 30 μ thickness were found most convenient, for thinner sections give too many broken rays, while spicule overlap obscures the optical phenomena to a great extent in hicker sections. Since even in 30 μ sections the oscular rays are bound to be broken (their tips are nearly 200 μ apart), it is essential to have the spicules irmly embedded, and the Peterfi celloidin-paraffin method was found to be atisfactory. The material used must of course be uncontracted, necessitating its immediate fixation after collection. A neutral 5 per cent. solution of ormaldehyde in sea-water was employed as fixative and the specimens were tored in 90 per cent. alcohol to prevent spicule corrosion. After dewaxing, he unstained sections were mounted in balsam or D.P.X.

Only unbroken basal rays which were in focus all along their lengths were elected for the measurements of optic angle, and in the case of the quadri-adiates there was the additional condition that the gastral ray had also to be unbroken and in the same focal plane as the basal ray. In this way it was ensured that the optic axis lay in a plane perpendicular to the axis of the microscope.

To obtain some idea of the form of the spicules occurring in the tube sectioned, part of the material was left uncut and, after dewaxing, temporarily mounted, choanoderm uppermost, in 90 per cent. alcohol. By suitably tilting he piece of wall and using a modified camera lucida (see p. 36), spicules from different levels could then be drawn in 'surface-view aspect', i.e. with the basal ay perpendicular to the line of sight and the two oscular ray tips in the same ocal plane of the microscope. Since all parts of the spicule were brought into focus before being drawn, the drawing represents the form of the spicule a projected into a plane perpendicular to the line of vision, and such drawing enable one to identify approximately the original position in the tube of subsequently isolated spicules, because the spicule form varies continuously wit the distance from the oscular edge. Furthermore they enable the optic angle measured to be correlated with the shapes of the spicules as seen in surfac view.

Isolated spicules

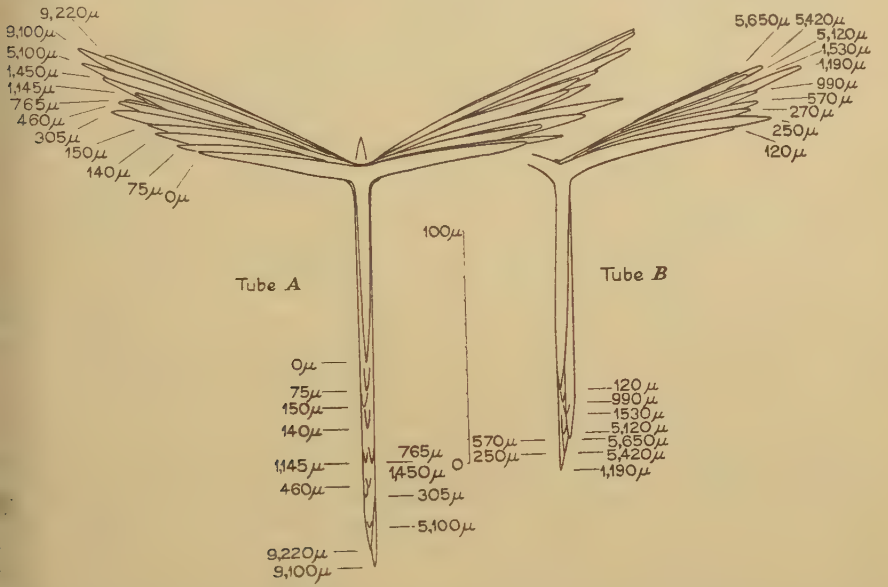
Spicules were isolated from several distinct pieces of wall by boiling for few minutes with 5 per cent. potash solution, washing several times in distille water with the help of a centrifuge, and then suspending the spicules in absolute alcohol and allowing a drop of the suspension to spread and dry on slide. Selected specimens were then picked up under a 1½-inch objective upo the tips (50 μ thick) of finely drawn glass needles which had been lightl smeared with balsam, and the needles were mounted on a slide by means o a piece of plasticine. The spicules must of course be taken up in a suitabl orientation to be of use, and the basal ray affords a convenient handle, sinc it enables the spicule to be set for both side- and surface-views if it is in lin with the needle. The surface-view aspects of the spicules could thus be draw as before, while their optic axes could be determined after setting them fo side-view observation. Confirmation of the measurements of optic angl derived from the sections, and of the correlation between optic angle an spicule form, was thereby obtained.

A modified camera lucida was employed for the drawings, to avoid change in magnification as the microscope tube was racked up and down. Th mirror of the manufactured article was removed and replaced by a large plan mirror which was clamped at an angle of 45° to the bench by means of a retor stand. Since the mirror was not attached to the microscope, nor moved relatively to it, the picture was always projected the same distance and hence th magnification factor was constant. This is important because excessive racking was often necessary to bring the isolated spicules, perched on their needles into focus in the required orientation. The arrangement must of course b distortionless and this was checked by drawing part of a squared eyepiec micrometer with the apparatus and then measuring the sides and angles of th drawing. A ×40 objective and a ×10 eyepiece gave the most convenien magnification.

Results

Appearance of spicules in surface-view

In fig. 1 superimposed drawings of the surface-view aspects of tri- and quadri-radiates derived from successive levels of two oscular tubes are depicted. It is obvious at a glance that the form and size vary continuously from the oscular edge to the base of the tube, and such an arrangement enables one to determine approximately the original position of subsequently isolated icules. However, only those spicules that are in contact with the choanoerm give such a consistent picture; if 'outer' as well as these 'inner' spicules re included, the arrangement is not quite so perfect. The reason is that the icules grow in contact with the choanoderm (Minchin, 1908) and leave it only hen fully grown, so that only the inner spicules are of similar ages and formed nder a given set of conditions; the outer spicules were formed earlier, when e dimensions and possibly the rate of growth of the tube were different.



IG. 1. Superimposed surface-view drawings of inner tri- and quadri-radiates of two oscular ubes, *A* and *B*, derived from different specimens of *L. complicata*. For each drawing the scular rays are symmetrically disposed on each side of the mid-line. The distances of the picules from the oscular edge are indicated. Note the dependence of the oscular ray curvature on the distance.

ptic angles of tri- and quadri-radiates

When the optic angles of tri- and quadri-radiates are plotted against the istance of the spicules from the oscular edge, the points form the pattern hown in fig. 2. At the edge itself high readings are obtained, but at a disance of only 200 μ behind they are several degrees lower, and thereafter ecline gradually towards the base of the tube. The results from two tubes *A* and *B*) are given to show the variation. The tubes were taken from specimens collected at Plymouth in different years and while the patterns of points re similar, that of tube *B* is about 3° lower than that of tube *A*, and a check f some of the measurements has shown that the difference is not due to a ystematic error of measurement. Three other tubes from the first specimen ave similar points to those of tube *A*, though their patterns are less wellefined because fewer readings could be taken; the number of spicules which atisfy the conditions for measurement depends on the orientation of the

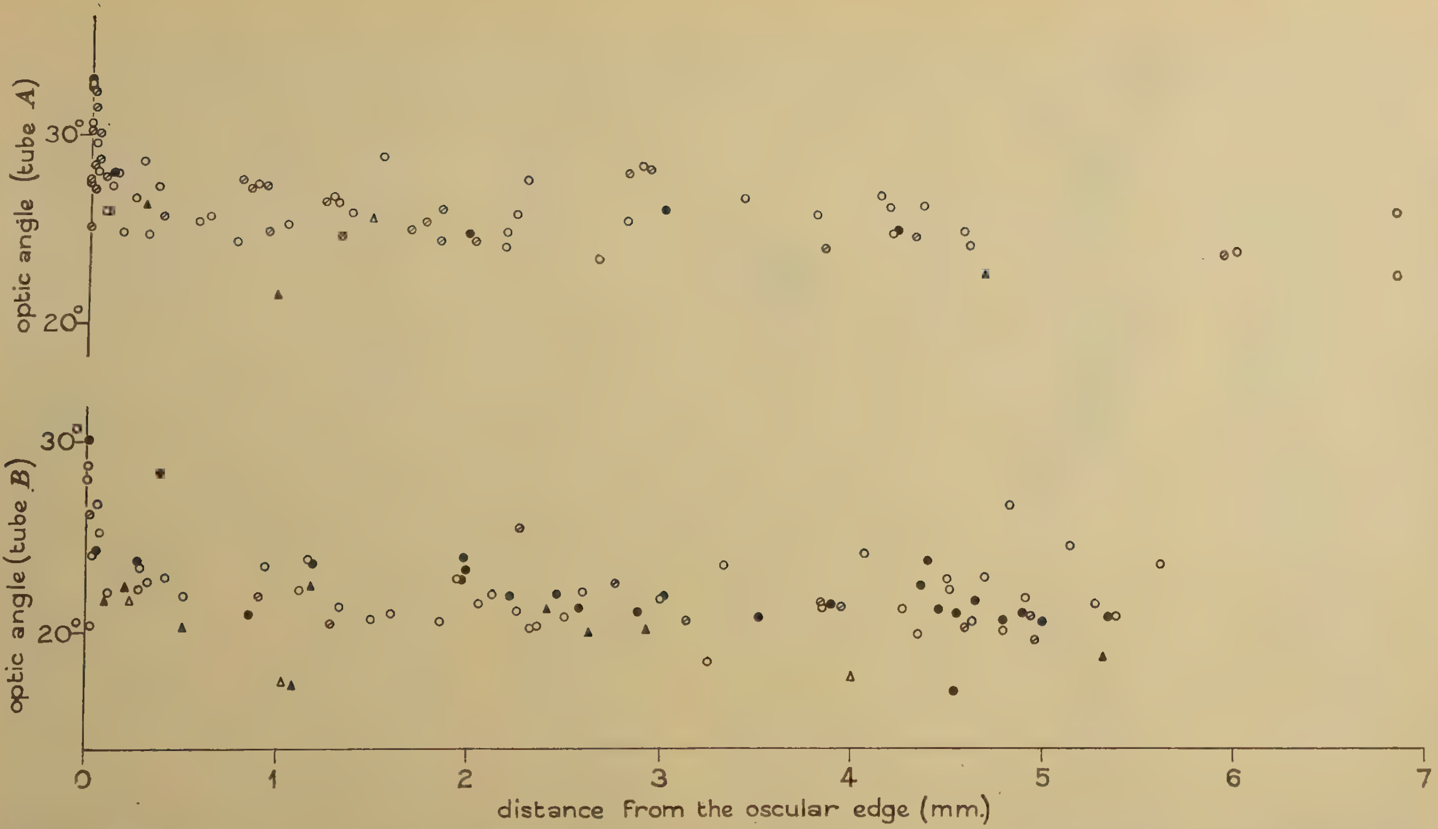
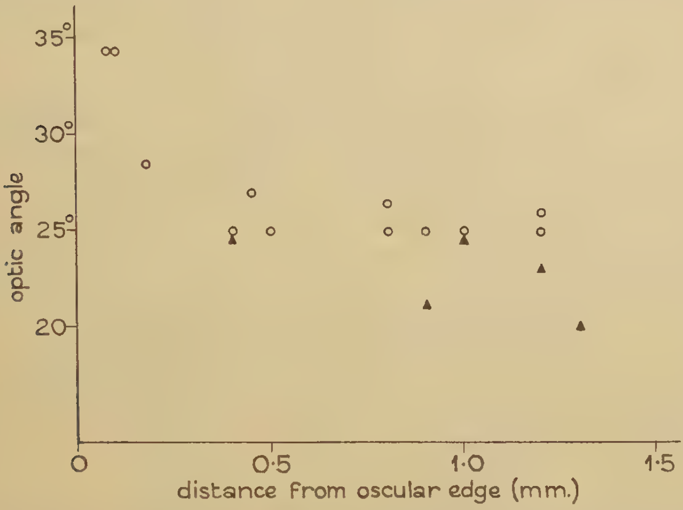


FIG. 2. Graphs showing the relation between the optic angle and the distance from the oscular edge for tri- and quadri-radiates of tube *A* (above) and tube *B* (below), as determined by using longitudinal sections of the oscular tube. Inner quadriradiates, ○; quadriradiates with basal rays less than 75 μ, ⊘; outer quadriradiates, ●; inner triradiates, △; triradiates with basal rays less than 75 μ, ◬; outer triradiates ▲

aterial for sectioning, and even with straight tubes it is not always possible orient the sectioning as accurately as desired. However, measurements made ith isolated spicules confirm the pattern, as indicated, for example, by the aph in fig. 3 (tube *C*). This tube was derived from the same specimen as be *A*.



IG. 3. Graph showing the relation between the optic angle and the approximate distance om the oscular edge for tri- and quadri-radiates of tube *C*, as determined by using isolated spicules. Quadriradiates, ○; triradiates, ▲.

It might have been expected that the surface-view drawings of the spicules f tubes *A* and *B* would reflect the difference between the two patterns of oints, but this is not the case (fig. 1). The drawings are quite comparable, he only important difference being one of size; the spicules of tube *B* are maller than those of tube *A*, a fact to be correlated with the smaller girth of he former tube. This was approximately 750 μ in diameter in the middle, vhereas tube *A*, which was elliptical in cross-section, had large and small iameters of 1,700 μ and 750 μ respectively in the same region. The similarity f the surface-view aspects and the opposing dissimilarity of the optic angles f tubes *A* and *B* reveal that the optic angle cannot be the sole factor influen-ing the curvature of the oscular rays, and this is supported by the fact that the orm changes markedly over the first 1,500 μ, whereas the optic angle has eached a comparatively steady level after only 200 μ. Furthermore, the study f isolated spicules has shown that two spicules with the same surface-view ppearance can have different optic angles, while two with the same angle can ave different shapes as seen in surface-view (fig. 4). The optic angle is hence ot closely correlated with the curvature of the oscular rays.

A considerable amount of scatter occurs on the graphs, which cannot be xplained by errors of measurement (made to within $\pm\frac{1}{2}°$), or by the fact that ome 'outer' spicules were measured, since these spicules give points evenly distributed amongst the scatter. Young spicules likewise do not give value consistently higher or lower than the rest, as the graphs indicate. The scatte can be partly explained by the formation of the spicules at different times an hence under possibly different growth conditions, but more so perhaps by th absence of a continuous well-defined reference line with respect to which th

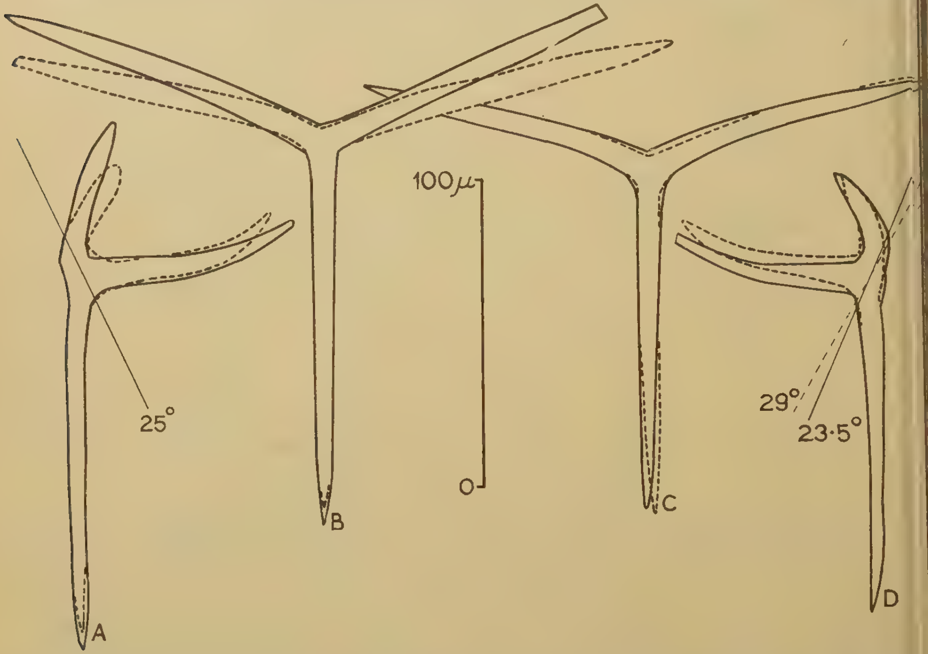
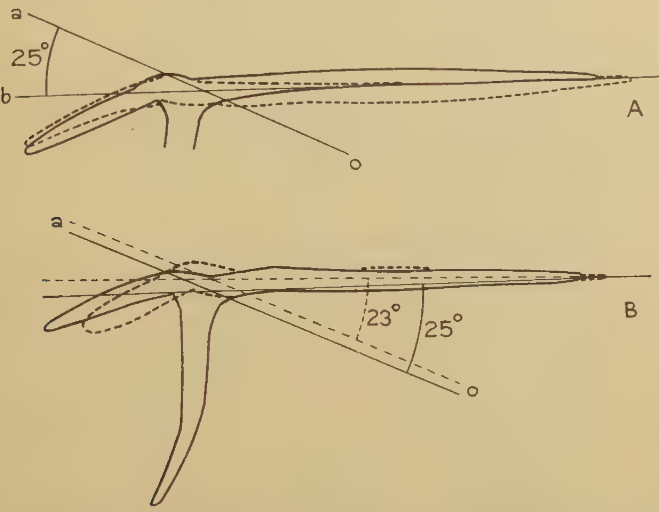


FIG. 4. Superimposed surface- and side-view drawings of two pairs of isolated quadriradiate selected to show that spicules with the same optic angle can have different oscular ray curvatures (A and B), while spicules with the same shape in surface-view can have different optic angles (C and D). Each pair of spicules was derived from the same oscular tube, and the two tubes were taken from the same specimen. The pair depicted in A and B correspond to spicules separated from the oscular edge by 400 μ and 1,200 μ respectively; that in C and D to spicules 300 μ from the edge. The optic angles are given in the diagram.

optic angle can be measured. The unevenness of the choanoderm and pinacoderm prevents the precise determination of the direction of the tube wall at each site, and the basal ray axis actually employed gives only a rough guide to this direction, because of the variability in curvature of the ray. Thus in quadriradiates the basal ray almost invariably curves with the concavity on the choanoderm side of the ray, and usually has a pronounced kink near the spicule centre (fig. 5, B). In triradiates, on the other hand, the kink is directed towards the opposite side, while the curvature of the distal part of the ray may be concave or convex towards the choanoderm (fig. 5, A). This variation in curvature is a reflection of the unevenness of the choanoderm since each ray is founded by a cell in contact with this layer.

The difference in curvature between tri- and quadri-radiates accounts to some extent for the low values of optic angle given by some triradiates (see gs. 2 and 3), for their basal ray axes do not coincide with the inner (gastral) lge of the distal seven-eighths of the ray, which would give a better indicaon of the direction of the tube wall; and their angles would be increased by or 2 degrees if this edge were taken as the reference line. In the quadridiates the corresponding edge does in fact coincide in most cases with the asal ray axis used. Thus in fig. 5, B the side aspects of a triradiate and a quadridiate from tube *C* having the same surface-view appearance (corresponding



IG. 5. A, superimposed side-view drawings of a tri- and quadri-radiate having the same ptic angle. The difference in curvature of the basal rays is typical. *oa*, optic axis; *br*, basal ay axis; triradiate in broken outline. B, side-view drawings of a tri- and quadri-radiate supernposed with the gastral surfaces of the basal rays coinciding. The optic axes are parallel, but ne measured optic angles differ by two degrees. The low values of optic angle for some of the riradiates plotted on figs. 2 and 3 would likewise be increased if measured with respect to the astral surface of the basal ray, which gives a better indication of the direction of the choanoderm. *oa*, optic axis; triradiate in broken outline.

o a position of 1,200 μ from the oscular edge) are superimposed so that the astral surfaces of the basal rays coincide; their optic axes have become arallel, but the measured optic angles were respectively 23° and 25° (as may e seen in fig. 3). It thus seems likely that the optic axes of tri- and quadriadiates of similar age and situation are alike and that the optic axis is directly elated to the direction of the tube wall.

There is a possible difference between tri- and quadri-radiates, however, in hat values for the former have never exceeded 28°, even when the angle is neasured against the gastral edge of the basal ray; but these spicules are unommon within 200 μ of the oscular edge (only three have been measured from his region) and possibly a sufficient number has not been examined.

urved monaxons

Lance-headed monaxons are stout, uni-rayed spicules which curve in a ingle plane containing the optic axis, as Sollas and von Ebner showed for various species. Because of the curvature, the optic axis makes a continuous varying angle with the direction of the ray, but at the lance-head (the fir formed part (Minchin, 1908)), it is directed at right angles (at least to withi 5°) to the axis of the tip. Fig. 6 shows some examples drawn from differen

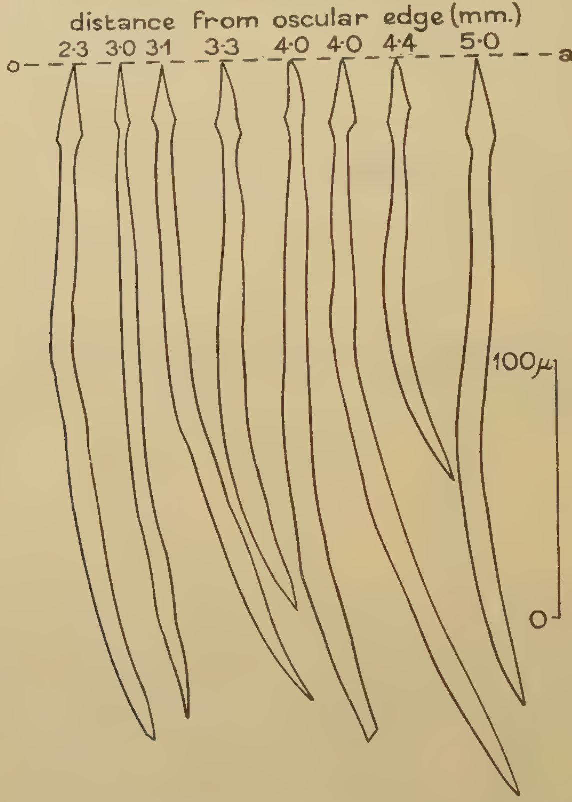


FIG. 6. Drawings of lance-headed monaxons belonging to the same oscular tube, showing tha the curvature is independent of the distance from the oscular edge (indicated by the figures and that the optic axis (*oa*) is nearly perpendicular to the axis of the lance-head.

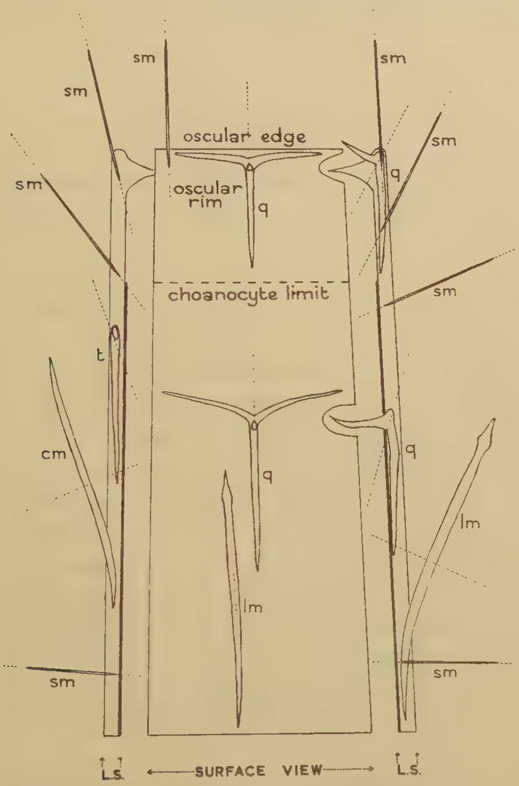
parts of an oscular tube. The curvature is independent of the distance fror the oscular edge.

The curved monaxons which lack the lance-heads, or have them poorl developed, have optic axes corresponding to those of the lance-headed type

Curved monaxons are oriented with their plane of curvature in an axia plane of the oscular tube and their free ends pointing away from the tube base (fig. 7).

Slender monaxons

The slender monaxons are straight, pointed at each end and slender, usuall with a slight thickening towards the embedded end. At and near the oscula lge they are oriented to form a crown, but over the greater part of the tube ey are haphazardly directed outwards. Possibly they tend to grow perpencularly to the surface, as Urban (1906) describes for *L. eleanor*, and are ibsequently disturbed by contractions (or fixation) of the tube. For syconoid ecies Woodland (1905) mentions that the monaxons are irregular below the scular rim, whereas Bidder (1898) states that the slender monaxons are erpendicular to the surface. Much would thus seem to depend on the bserver and the conditions under which the material has developed or been xed.



IG. 7. Diagram summarizing the orientation of the spicules and their optic axes (shown as roken lines) with respect to an oscular tube of *L. complicata*; *sm*, slender monaxon; *cm*, urved monaxon without well-defined lance-head; *lm*, curved monaxon with lance-head; *q*, quadriradiate; *t*, triradiate; L.S., longitudinal section.

In the slender monaxons of *L. complicata* the optic axis coincides exactly ith the axis of the spicule, and Bidder (1898) records a similar observation or the slender monaxons of *Sycon raphanus*. Von Ebner's statement (1887) hat in all monaxons the optic axis is inclined to the spicule axis is thus ncorrect.

The diagram in fig. 7 summarizes the orientation of the spicules and their ptic axes.

DISCUSSION

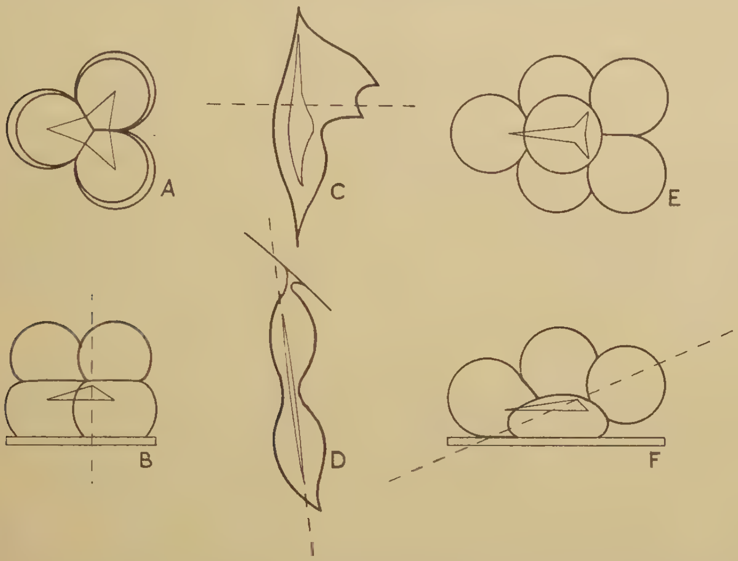
Before discussing the mechanism whereby the optic axes become oriente a brief account of spicule development and structure are necessary. According to Minchin (1908, 1909), each spicule ray (monaxons included) secreted by a pair of cells, which probably arise by the division of a calcobla mother-cell. The basal and oscular rays of tri- and quadri-radiates are thu formed by a sextet of cells, of which three are in close contact with the choanoderm. Spicule formation begins with the secretion of three non-birefringen rodlets, each between a pair of cells, which join up centrally to form a three-rayed primordium upon which calcite is subsequently deposited. The inne cell of each pair then acts as 'founder', secreting material at the tip of th developing ray, while the outer one only later passes along the ray, thickenin it as it proceeds. For monaxons the development is essentially similar to tha of a ray of a triradiate.

As regards spicule structure, Minchin and Reid (1908) have shown that th spicules of *L. complicata* (and other species) contain along the centre of eac ray a stainable axial filament, which remains when the calcite has been corroded away. This settles the old controversy, summarized by Minchin (1909) between von Ebner, who concluded that the filament was an optical illusio caused by the presence of a central strand of less pure calcite, and Sollas (fo example), who believed in the presence of organic matter, largely because th spicule chars internally when ignited. Further evidence is afforded by Maa (1904), who decided that an organic network permeated the whole spicule substance, because concentrated potash solution breaks up the spicule into minut calcite rhombohedra, which he believed were liberated by the dissolution o the organic matter holding them together. He also found (1906) that whe syconoid sponge larvae were grown in sea-water containing no lime, calcareou spicules were not formed but were replaced by organic 'spiculoids', secreted b the same type of cell in approximately the same triradiate or needle-like form Such spiculoids suggested an over-secretion of the organic matter of the norma spicule in the absence of calcium carbonate. All these facts support the conclusion that organic material is present.

Mechanism of orientation of the optic axis

In *Leucosolenia* [= *Clathrina*] *coriacea* the sextet of formative calcoblast has a regular arrangement (Minchin 1898), three of the cells being exactly superimposed on the other trio in contact with the choanoderm. The triradiates formed by these sextets are 'perregular' (von Ebner, 1887), i.e. have equal rays meeting centrally to form three equal angles and an optic axis set perpendicular to the plane of the three ray tips; and since the optic axis is almost at right angles to each ray and one ray points to the base of the tube (as in *L. complicata*), it follows that the optic axis is very nearly perpendicular to the surface of the tube. The optic axis thus coincides closely with the trigonal axis of symmetry of the sextet (fig. 8, A and B).

In *L. complicata*, on the other hand, the arrangement of the calcoblasts is
scribed as irregular by Minchin (1908), but a close examination of Minchin's
vn drawings in this paper indicates that in four sextets out of the five por-
ayed (plate 17, nos. 19, 20, 21; plate 20, no. 90) the outer three cells are dis-
aced forwards (i.e. towards the osculum) with respect to the inner trio, as
own by the orientation of the contained spicule primordium. Such a dis-
acement would have tilted the axis of the sextet forwards (see fig. 8, E and F)



IG. 8. Diagrams derived partly from Minchin's drawings of spicule development showing
ie tendency for the optic axis of the first-formed calcite rudiment to lie in the direction of
ell division (or cell association). A, B, surface- and side-views respectively of a sextet of
eucosolenia [= *Clathrina*] *coriacea*; E, F, the same for *L. complicata*; C, early stage in the
ormation of a lance-headed monaxon; D, the same for a slender monaxon. The optic axis is
indicated by the broken line.

nd if once again the optic axis of the calcite coincided with this axis, the optic
xis would have the orientation found in the developing tri- and quadri-
adiates of this species. Minchin's own description of the arrangement as
rregular could have arisen from disturbance in some cases during the process
f fixation, or more probably, from the lack of sufficient time for some young
extets to become oriented before fixation. The presence of gastral ray calco-
lasts beneath many of the sextets may also have confused the picture to some
xtent.

For the monaxons the optic axis also tends to coincide with the long axis
f the cell pair (fig. 8, C and D). This is not so evident in the one case depicted
y Minchin for the lance-headed monaxon, but the calcite mass is quite large
n this case, and the arrangement of the cells suggests that at an earlier stage
n development the tendency would be more marked. A law can thus be
entatively stated: for spicules of *Leucosolenia coriacea* and *L. complicata* the

optic axis of the first formed calcite rudiment lies in the direction of the c
division (or cell association) that directly precedes its formation.

Two outstanding problems remain, namely, how does the optic axis cor
to coincide with the axis of the sextet, and how is the outer trio displac
forwards relative to the inner trio in *L. complicata*? As regards the latt
experimental evidence in support of a hypothesis of 'jelly-shear' has be
obtained by the author (Jones, 1952) and will be published shortly. Brief
the sextet is anchored to the inner cell-layer by the inner trio, and is orient
and tilted forwards by the slow shearing creep of the viscous mesogloeal jel
past it, arising from the secretion of the jelly in the more basal feeding par
of the tube (the choanocyte zone) and the extension by growth of the inn
cell-layer at mainly the oscular end of the tube. The variation in the opt
angle with position in the tube is the result of the different amount of shearin
at each site, depending on the relative rates of growth in the different region
while the difference between *L. coriacea* and *L. complicata* sextets is accounte
for by the much slower growth of the oscular tubes of the former as compare
with the latter (Urban, 1906); the shear would hardly be appreciable durin
the early stages of spicule development after the formation of the sextet
L. coriacea.

The problem of the coincidence of the optic and trigonal sextet axes ca
largely be solved by considering the organic constituent of the spicul
Minchin (1898) observed that the first lump of calcite is deposited upon th
non-birefringent rudiment, later identified with the axial filament, and it seem
plausible to assume that the calcite ions are chemically bonded to those of th
precursor. The orientation of the calcite optic axis would then depend on th
crystalline structure of the precursor and the way this is oriented in the cell
sextet, and there seems no reason why it should not be appropriately oriente
in the direction of the prior cell division, particularly as the mitotic spindl
and the presumed cell membrane between the daughter cells are oriente
structures capable perhaps of functioning as precursors. Oriented over
growth is a well-established process. Thus Schmidt (1932*b*) gives a diagram
of oriented crystals of calcium iodide grown on a mica plate and remarks tha
crystalline dye particles can be oriented by the 'righting power' of the micella
on which they crystallize. Oriented crystals of (organic) urea can also form o
the cubic crystals of (inorganic) ammonium chloride.

Without the concept of oriented overgrowth it is difficult to understan
how the first-formed calcite primordium is oriented and held in the requisit
orientation. Schmidt (1932*a*) has given a brief review of the relation betwee
the optic axis and spicule form, and points out that mechanical orientin
agencies can act solely by virtue of the form and are not concerned with th
optical properties of the material, while, on the other hand, though an electro
magnetic field could orient the optic axis, it would not directly influence th
form. Hence Schmidt comes to the view that there must be a relation betwee
the form and the optic axis, depending on a tendency for the mineral to crystal
lize more easily in certain directions. Thus within the calcoblast (he refer

re to echinoderm spicules) the growth of calcite apparently goes on most sily in a direction perpendicular to the optic axis, and the branching of the ys at 120° is also a consequence of the properties of the crystallizing sub- ance; should, therefore, mechanical conditions set the spicule, the optic axis ould likewise become oriented, or should the optic axis be directly oriented, would become the form also.

Now the assumption that the crystallizing activity of calcite determines the lation between the optic axis and the spicule shape cannot apply to sponge icules, for, as shown above, the variation in spicule form is not closely corre- ted with the variation in optic angle. The basal ray of *L. complicata* develops ecociously (Minchin, 1908) and could perhaps be oriented by mechanical ctors, but the angle which the optic axis makes with this varies continuously om about 18° to 35°, and hence surely cannot be determined by the process crystallization alone, since this generally involves development in the direc- on of particular crystal faces or corners. To assume that slight variations in e composition of the solution bathing the ray tip are responsible for such ight differences in the direction of growth relative to the optic axis seems ntenable, especially as the solution would not only have to vary with the posi- on of the sextet in the oscular tube, but also during the development of the icule itself, for the basal ray is often kinked.

The alternative suggestion, that an electromagnetic field orients the crystal rimordium, is likewise incredible, because, while bio-electric gradients are elieved to exist in species of *Leucosolenia* (Hyman and Bellamy, 1922), they o not nearly approach the large field strengths required for the orientation f crystals of polaroid, say, or for even the imperfect orientation of molecules f glass or certain liquids that can be obtained by using a Kerr cell.

It may be concluded then that the optic axis is oriented, not by the bio- lectric field or the crystallizing activity of calcite, but by a process of oriented vergrowth upon an organic precursor which is structurally bound and riented within the formative cell complex. The cells and precursor can per- aps be regarded as a single complex organic crystal within which the calcite chemically bonded so that its optic axis lies in the direction of the cell divi- ion (or cell association) preceding its secretion; the orientation of the optic xis relative to the sponge wall then arises from the orientation by mechanical rocesses of the formative cell complex.

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The Deposition of the Third Instar Larval Cuticle of *Calliphora erythrocephala*

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With one plate (fig. 1)

SUMMARY

histological study has been made of the formation of the cuticle of the third ar of *Calliphora*.

ncrease in size of the epidermal cells and nuclei, particularly in the spiny regions, ceded the release of the moulting fluid. The cytoplasm first became vacuolated this was followed by the appearance of many granules. The basement membrane onstituted in part by stellate tracheoblast cells. The membrane breaks down just ore cuticle deposition and reappears after ecdysis.

he outer epicuticle is formed from coalesced droplets aligned at the cell surface to m a delicate continuous, folded, acidophil, lipoprotein layer. Further, protein and oid (cuticulin) is added beneath this layer from cytoplasmic processes to form the er epicuticle. These processes later became the pore canals. Endocuticle is secreted filaments from the epidermal cells between and around the processes.

uring moulting, phenolic substances and oxidase are transported to the inner cuticle only in the cuticular spines. Wax and cement layers are not formed and re are no dermal glands.

he peak in the secretory cycle of the oenocytes corresponds to the time of deposi- of the epicuticle and the secretory material shows similar staining properties to cuticular material. The oenocyte groups are connected with each other and with epidermis by cytoplasmic extensions, through which secretory granules pass.

he muscle insertions are not attacked by the moulting fluid. They are thought to tain protein-bound sulphur. The separation of the muscle insertions and sensory ans from the exuvial cuticle occurs just before ecdysis.

he nature of the epicuticle and the theory that the oenocytes are associated with formation is discussed.

INTRODUCTION

RECENT studies have shown that the insect cuticle is a complex, highly differentiated, and variable association of chitin, protein, and lipoid, pregnated with various materials depending upon the insect species, the rticular integumental region, and the developmental period. Considerable iation in the composition and number of layers within the epicuticle has en recorded for different species (Dennell, 1946; Webb, 1947; Wiggles- rth, 1947; Richards, 1951; Malek, 1952; Dennell and Malek, 1953). idence for the existence of different epicuticular layers is best obtained by lowing stage by stage the histological changes taking place in the epidermis

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and its associated glands during the deposition of a new cuticle. Studies this nature have so far been made with only a few insect species; e.g. *Rhodn prolixus* Stahl (Wigglesworth, 1933, 1947), *Tenebrio molitor* L. (Wiggleswor 1948), *Diataraxia oleracea* L. (Way, 1950), *Ephestia kuhniella* K. (Kühn a Piepho, 1938).

Apart from short descriptions of the histological changes in the epider during the moulting of the larva of *Calliphora vomitoria* L. (Willers a Dürken, 1916) and *Calliphora erythrocephala* M. (Hoop, 1933) there hitherto appeared no account of the deposition of the dipterous larval cutic Quite extensive studies have been made of the physical and chemical propert and the histology of the third instar cuticle of *Sarcophaga falculata* Pa and *Calliphora* immediately before and during puparium formation (Fraenl and Rudall, 1940, 1947; Pryor, 1940; Dennell, 1946, 1947). The nature the associations between epicuticular lipoids and proteins in the third ins cuticle of *Calliphora* have been studied by Hurst (1948, 1950) who has forward an epicuticular valve model in an attempt to explain the asymmetri behaviour of the cuticle to water transmission, lateral diffusion of solutio within the epicuticle, and the penetration of polar and non-polar solvents

It was felt that knowledge of the deposition of the third instar larval cuti of *Calliphora* was required before comparisons could profitably be made wi the cuticle of other insects.

MATERIAL AND METHODS

Larvae of *Calliphora erythrocephala* Meigen were reared from unretain eggs deposited by 7-day-old flies on thin slices of lean horse-meat in cover Petri dishes in an incubator at 24° C. and 70 per cent. relative humidi Only eggs that had been deposited within an hour's interval were used initiate larval cultures. Each culture contained 75–100 eggs. The use of th meat slices facilitated periodical examination under a binocular microsco to determine the developmental stage. Regulated amounts of meat were add when the larvae reached the third stadium. When the larvae were 60 hou old the Petri dish covers were removed from the cultures and the dish placed in a pan containing a layer of sawdust into which the fully gro larvae migrated approximately 36 hours before puparium formation. Larv reared on sterilized synthetic media or autoclaved brain were unsuitab because of the great variation in the growth-rate.

Larvae punctured anteriorly or posteriorly were fixed in formol-sali (Baker, 1949) or Flemming without acetic in *Drosophila* Ringer. Peterf celloidin-paraffin embedding procedure (Pantin, 1946) gave the best resu although good paraffin sections could be obtained after isopropyl alcoh dehydration followed by clearing in cedarwood oil or terpineol and embeddi in paraffin (m.p. 60° C.). The staining procedures used were Heidenhai iron haematoxylin followed by orange G, Mallory's triple stain, Man methyl blue eosin, and gallocyanin counterstained with erythrosin. The la procedure gave excellent differentiation of the inner epicuticle and po als. It was also useful for studying the cytoplasmic basiphilia of the epi- mis. Ehrlich's acid haematoxylin counterstained with eosin and Giemsa's n were used for staining whole mounts of the cuticle and epidermis. For study of lipoids, 10–15 μ thick frozen sections of formaldehyde-calcium d tissue, infiltrated with gelatin at 35° C., were prepared and stained with lan IV, sudan black B, or nile blue according to the techniques recom- nded by Kay and Whitehead (1935), Baker (1944, 1949), and Cain (1947). e quality of the sudan black was found to be very important and better ults were obtained when a pre-war stock of Meyer's product was used. toplasm extending into the pore canals and the canals themselves were best died after fixation in 1 per cent. osmium tetroxide and lightly counter- ined with orcein. All frozen sections were mounted in Farrants's medium. ase-contrast microscopy of unfixed tissues was useful for the clear ferentiation of the oenocytes and the larval cuticle. Various histochemical ts have been used and these are mentioned, as well as special histological hniques, at the appropriate places below.

owth of the larva

Under the rearing conditions described, the approximate development nes for the larva and pupa are shown in Table 1.

TABLE 1. *Growth-rate of the larvae*

Developmental period	*Duration*
iposition to hatching	22 hrs.
rst stadium	16 ,,
cond stadium	24 ,,
hird stadium—larvae feeding, crop full	40 ,,
hird stadium—larvae not feeding, crop emptying	24 ,,
hird stadium—resting period in sawdust 'Prepupal period' of Wardle; mature larval period of Fraenkel, 1935	36 ,,
Vhite puparium' to extension of pupal horns—prepupal period	25 ,,
pal	10–11 days
TOTAL	18–19 days

The individual variation in the growth-rate during the short larval period 6 days made accurate time marks of the growth stages during the moulting cle impossible. By counting the number of cast skins appearing on the rface of the thin meat slices and examining the anterior and posterior iracles of the larvae, the number that had moulted from the first to the cond or the second to the third instars could be determined. Sixty per cent. the larvae shed their cuticles over a 2-hour period, and within 3 hours early all larvae were in the succeeding instar. The description of the moulting cle is confined to the moult from the second to the third instar. The term cdysis' refers throughout to the actual shedding of the cuticle. The term noult' has a wider meaning including the deposition of the new cuticle.

OBSERVATIONS ON THE LARVAL MOULTING CYCLE

The epidermis

The larval epidermis consists of a single layer of large, cuboidal, or flatten cells of uniform appearance. In the Cyclorrhapha most purely larval orga grow by increase in cell size, whereas presumptive imaginal organs grow b cell multiplication. Trager (1935, 1937) points out that those tissues destine to be histolysed and replaced at metamorphosis grow by increase in cell siz consequently, there are no crises of mitosis or chromatolysis preceding th formation of the new cuticle in *Calliphora*. The 'chromatin droplets' regarde by Wigglesworth (1942) as resulting from the dissolution of superfluou nuclei after the mitotic period in *Rhodnius* have not been observed in th epidermis of *Calliphora* before moulting. The rapid increase in cell size i the third stage larvae results in a change from cuboidal to hexagonal outlin of the cells. They increased in volume from approximately $350\mu^3$ (nucleu $80\mu^3$) in the first stadium to $10{,}000\mu^3$ (nucleus $2{,}000\mu^3$) in the newly moulte third instar larvae and then dramatically to $250{,}000\mu^3$ (nucleus $8{,}000\mu^3$) i the full-grown larva. Wagner (1951) has shown graphically this tremendou increase in nuclear volume. Besides the overall increase in cell and nuclea size, there was a subsidiary cycle of increase and decrease at each larval moul Feulgen staining showed a rapid increase in chromatic material in the nucleu up to the time of cuticle secretion and this paralleled the increase in nuclea size. A marked vacuolation (fig. 1, A) appeared at the base of cells in thi

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FIG. 1 (plate). (The figures are all photomicrographs.)

A, epidermal cells in surface view of the second instar larva, showing the vacuolation of th cytoplasm before the release of the moulting fluid. Giemsa.

B, transverse section of larval cuticle approximately 4 hours before puparium formation Hotchkiss PAS technique. Only the epicuticle and outer endocuticle were stained.

C, transverse section through the second instar cuticle, showing the moulting fluid space a it appears when first formed. Haematoxylin.

D, transverse section through folds, showing the cytoplasmic processes extending up to th new epicuticle. The epicuticle has separated from the epidermal cell surface during sectioning Mallory's triple stain.

E, a group of oenocytes from the second instar, showing the dense, finely granulated cyto-plasm and well-defined nucleus and nucleolus. Tracheoles can be seen ramifying over th cell membranes. Phase-contrast; cells in Ringer.

F, to show the relationship between the oenocytes and the epidermal cells. The connexion to the epidermis are clearly shown. Romano's silver technique.

G, to show a protoplasmic connexion between two oenocytes. Ehrlich's haematoxylin.

H, a transverse section through the cuticle and oenocytes of a late second instar larva showing the spaces in the oenocyte cytoplasm appearing after treatment with lipide solvents Haematoxylin and eosin.

I, a longitudinal section through the newly formed epicuticle, showing the cytoplasmi processes extending through the thin endocuticle. The cuticle layers have become separate during sectioning. Mallory's triple stain.

J, an oblique section through the cuticular spines of a newly moulted third instar larva showing the acidophil strand from the epidermal cell extending to the tip of the spine Mallory's triple stain.

K, a transverse section through the muscle insertion during deposition of the new cuticle showing the connexion of the tonofibrillae across the exuvial space. Mallory's triple stain.

L, cuticle surface of a newly moulted third instar larva after immersion in ammoniaca $AgNO_3$. The black spots represent the pore canals.

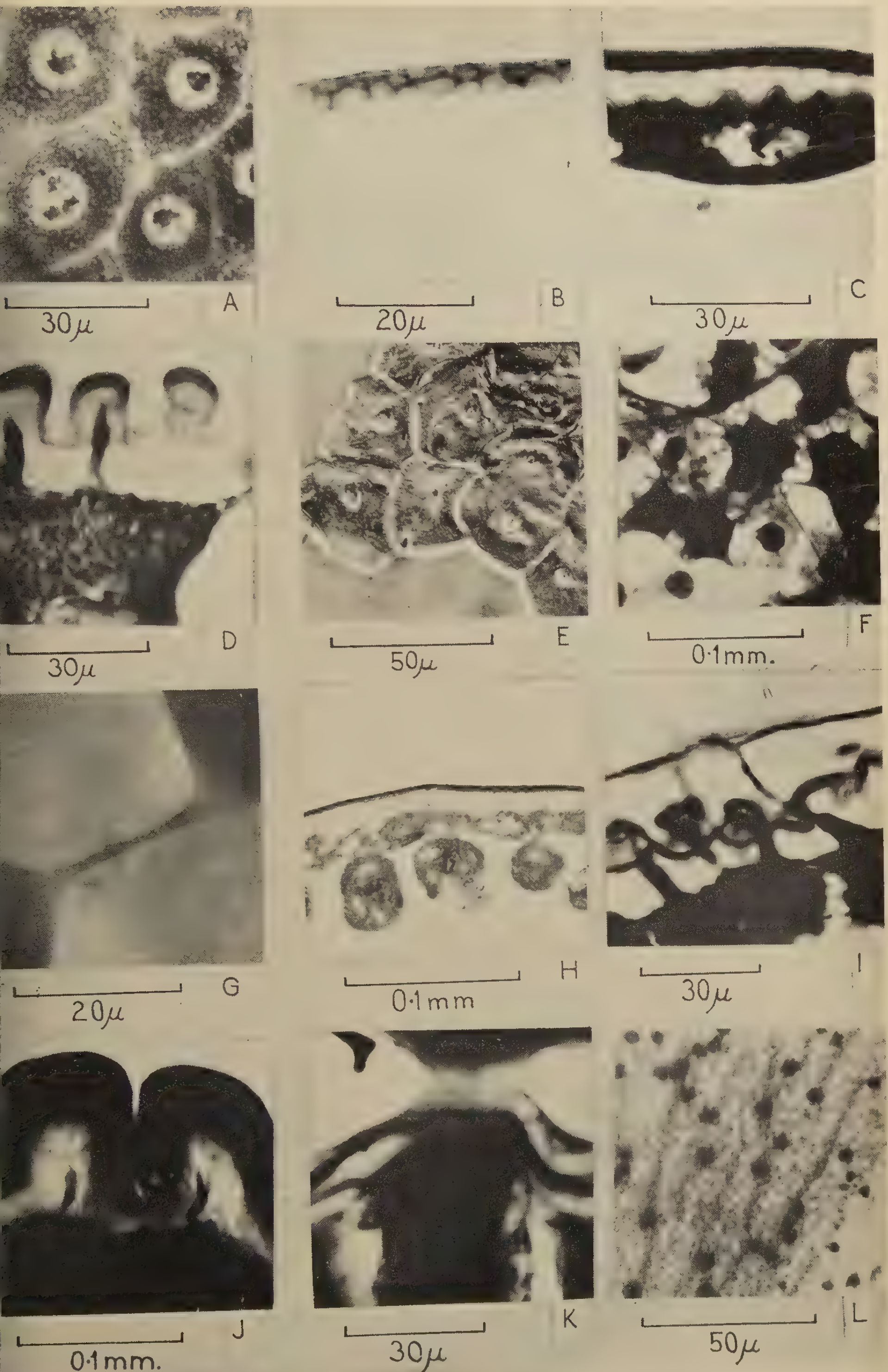

FIG. 1

L. S. WOLFE

ndition, as was also observed by Willers and Dürken (1916). Nuclear aterial was very diffuse in the intermoult period but became condensed just efore cuticle deposition.

Frozen sections of the body wall examined under phase-contrast showed e outer region of the epidermis to be syncytial. In formol-saline fixed aterial cell boundaries were found only in the lower part of the cells. Fixa- on in alcoholic solutions produced shrinkage and, hence, did not give a true icture of cell relationships. The epidermal cells were not prolonged into ails' or pointed and attached to the basement membrane as in *Rhodnius* Vigglesworth, 1933) and *Ephestia* (Kühn and Piepho, 1938).

The histochemical reaction of Hotchkiss (1948) for polysaccharides was erformed on the epidermis of larvae before, during, and after the formation f the new cuticle. PAS positive granules were found in each case, but the nlarged cells immediately before and during cuticle secretion reacted more ntensely. The epicuticle of the newly formed cuticle did not stain, but the picuticle of larvae immediately before puparium formation stained a bright ink or red (fig. 1, B).

The red staining material in the epidermis during the secretion of endo- uticle may be due to a polysaccharide material. The endocuticle, however, PAS negative. Richards (1952) demonstrated a Hotchkiss reaction in the picuticle in several insect groups and regarded it as indicating the presence f polysaccharides in the epicuticle. Brunet (1952) criticized the specificity of e test and pointed out that phenolic substances are also PAS positive. henolic substances are absent in the newly formed cuticle of *Calliphora*, but re present in the mature larval cuticle (Pryor, 1940; Dennell, 1947). It is hought that the staining in the late larval period is due to these substances.

Epidermal cells stained with Mallory's stain showed in the intermoult a nely, rather sparsely granulated, faintly acidophil cytoplasm. The size and umber of granules increased immediately before the release of the moulting uid and they accumulated at the base of the cells along with the appearance f many vacuoles. A pronounced basiphilia was observed after gallocyanin aining. Lagerstedt (1947, 1948) showed that the basiphilia, after staining ith gallocyanin, corresponded with the distribution of nucleic acid associated ith protein.

In the upper part of the cells just before the first signs of moulting, the asiphilia was replaced by an intense acidophilia particularly around the cyto- lasmic extensions into the second instar endocuticle. This condition was aintained until ecdysis. Increase in cytoplasmic basiphilia becoming re- laced by an acidophilia is a characteristic feature of glandular cells during reparation for secretion.

he basement membrane

An extremely fine, continuous membrane richly supplied with tracheae nd tracheoles was found immediately beneath the epidermis. Large stellate ells containing tracheoles of diameters 2μ and less were found within the membrane and could be distinguished readily by examination under phas contrast (fig. 2). No haemocytes were found attached to the basement membrane in the second instar or feeding third instar larva. However, larg numbers of blood cells were found up against the basement membrane i the posterior segments of the resting third instar larva. The basement membrane is continuous with the sarcolemma as noted by Pérez (1910) and Och (1946). Lowne (1893–5) recorded th absence of a basement membrane in th larva of *Calliphora*.

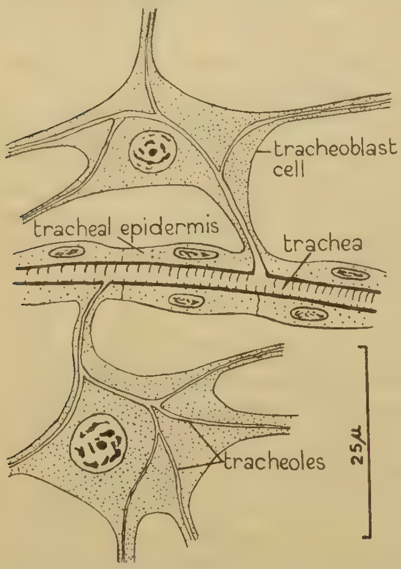

Fig. 2. The stellate tracheoblast cells from the inner surface of the epidermis of a larva in the second instar.

No such epidermal cell-tails as occu in *Rhodnius* (Wigglesworth, 1937) hav been found attached to the basemen membrane, and it is unlikely that th epidermal cells are connected with i formation as suggested by Lazarenk (1925). It also seems improbable that is formed from phagocytic blood cells o haemocytes. The great degree of trache lation and the numbers of stellate cell within the membrane in the second insta and particularly in the third instar larv suggests that processes of tracheobla cells at least take a part in the constitution of the basement membrane. Maye (1896) found stellate cells beneath th epidermis in the caterpillar and regarde them as secreting the membrane. Keiste (1948) has shown in *Sciara coprophil* that the tracheoles often develop independently from the tracheae, originatin as cytoplasmic canals within the tracheoblast cells and subsequently makin connexion with the tracheae. Processes from these stellate cells have bee observed to form a membrane in the honey-bee by Dreher (1936).

A basement membrane could not be found in the epidermis at the time o secretion of the third instar cuticle. The membrane appeared to have broke down. This is in direct contrast to the observations on *Bombyx mori* by It (1951), who stated that the basement membrane became decidedly thicker a moulting. The number of tracheoblast cells beneath the actively secretin epidermis in *Calliphora* markedly decreased and the cytoplasmic connexion of the oenocytes to the epidermis previously penetrating the basement membrane could not be seen directly connected to the epidermal cells. It is though that the tracheoblast cells degenerate at the moult and are re-formed fro tracheal epithelial cells in a similar way to that observed by Keister (1948) Observations on the basement membrane in *Calliphora* favour the hypothesi that it is constituted at least in part from processes of stellate cells whic break down and are reconstituted during the moulting cycle. The presenc membranes formed from tracheoblast cells around the internal organs of sects may possibly be a general phenomenon.

e liberation of the moulting fluid

Approximately 5 hours before ecdysis the vacuolation of the epidermal toplasm was succeeded by a granulation, and a small space was observed tween the epidermis and the endocuticle (fig. 1, c). This is interpreted as arking the release of the moulting fluid and the start of the dissolution of e cuticle.

The fluid is presumably secreted by the epidermal cells. Dermal glands are sent except the specialized wax-secreting peristigmatic glands (Keilin, 44). The cytoplasmic extensions into the pore canals which at first extended ross the exuvial space were not withdrawn, but the endocuticle was dis- lved around them and finally dissolved the pore canal contents. There was o indication of a new cuticle secreted over the epidermal surface at the time f first appearance of the exuvial space; the outer cell border, however, con- sted of many fine, closely aligned, acidophil and sudanophil granules. The uter epidermal cytoplasm stained intensely with sudan black, sudan IV, acid uchsin, and iron haematoxylin. The lipoid content of the cell border is robably the operative factor in the resistance of the cells to the moulting uid enzymes.

he secretion of the epicuticle

The granules aligned at the cuticle cell border after the liberation of the oulting fluid coalesced to form an extremely thin continuous sudanophil nd acidophil membrane. This layer also stained orange-red with Mallory's tain, black with haematoxylin, and pink with gallocyanin-erythrosin. No olour reaction could be detected with Millon's reagent. Three to four hours efore ecdysis, folds developed. They were produced by the extension of the uter cell surface assisted by contractions of the segmental muscles. They ere more readily seen in sagittal sections. The folds did not appear suddenly ut continued to form as further cuticle was being secreted up to the time f the first appearance of the endocuticle (fig. 3, A–D). Hoop (1933) did not ind genuine folds in *Calliphora* larvae during moulting. He assumed that the ncrease in size at ecdysis was due purely to stretching of the epidermis and uticle by air or fluid imbibition.

Acidophil and sudanophil filamentous strands, consisting of many small granules, extended from well within the epidermal cells up to the thin outer membrane and into the folds (fig. 3, c). A lipoid staining material was deposited from these filaments beneath the outermost layer. This material stained intensely with acid fuchsin and gave a faint pink reaction with Millon's reagent and purple with ninhydrin. The surface of the cuticle at this stage was soft, sticky, and very permeable.

The epicuticle is visualized as formed initially from a delicate, condensed,

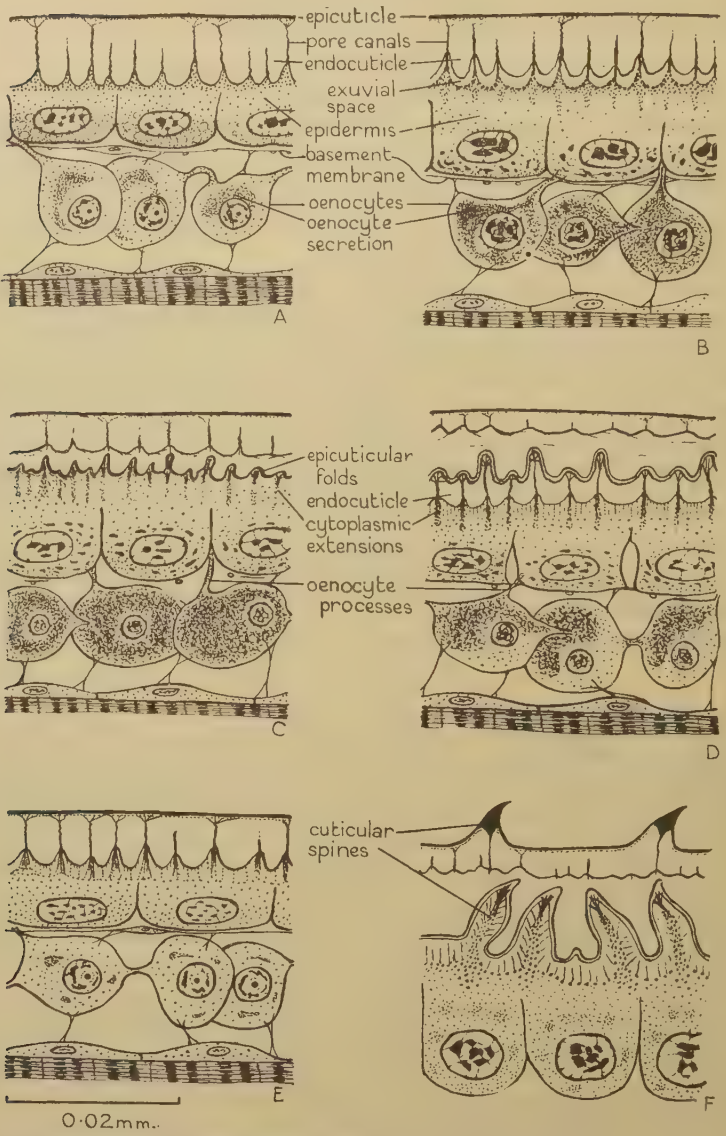

FIG. 3. The formation of the third instar cuticle of the larva of *Calliphora*. Semi-diagrammatic drawings of transverse sections through the developing cuticle, epidermis, and oenocytes. A, about 9 hours before ecdysis. B, about 5 hours before ecdysis; the first appearance of the exuvial space. C, about 3 hours before ecdysis; the formation of the epicuticle. D, 1–2 hours before ecdysis; the first deposition of the new endocuticle. E, immediately after ecdysis. F, formation of the cuticular spines.

po-protein surface layer separating the epidermis from the moulting fluid; ter, folds appear and there is a further deposition of epicuticular material om fine fibrillar processes extending out of the epidermal cytoplasm to eneath the outermost layer and adding to its thickness. The cytoplasmic sociation with the epicuticle was maintained throughout the moulting eriod. The filamentous strands from which the inner layer of the epicuticle as secreted later became the pore canals (fig. 1, D). Although the staining eactions of (1) the material in the outer epicuticle layer formed by coalescing f droplets on the surface of the epidermis, and (2) the outer epicuticular yer formed from the cytoplasmic filaments, were very similar, yet the outermost layer appeared more condensed and did not have the fibrillar structure f the inner layer.

Histological observations on the development of the cuticle indicate a two-yered epicuticle.

he changes in the oenocytes during moulting

The oenocytes were found as lateral clusters of twelve to fifteen irregularly haped cells immediately beneath the epidermis in segments 1–7 of the bdomen of the larva. Each cluster was richly supplied with tracheae and racheoles. The cells were approximately 25μ in diameter in the late second nstar larva with a nuclear diameter of 8μ and with one or two well-defined ucleoli (fig. 1, E). The oenocytes did not divide or degenerate during moulting but increased in diameter to 75–80μ in the third instar. During the ecretory activity of the oenocytes at the second instar moult the nuclear iameter varied and the nucleolus was not easily discernible. The cells were onnected to the basement membrane and to the sarcolemma surrounding he dorso-ventral muscles by delicate strands containing tracheolar branches. These strands supported and maintained the position of the cells during the muscular movements of the larva. Besides these supporting connexions, there were larger extensions of the oenocytes that penetrated through the basement membrane to the epidermal cells (fig. 1, F). These extensions contained cytoplasm and were more visible just before moulting and puparium formation. The oenocytes were also protoplasmically connected to each other (fig. 1, G). Each oenocyte cluster is, therefore, a syncytium, protoplasmically connected to the epidermis from which the cells were originally differentiated. In the rst instar larva the oenocytes were a multi-nuclear mass and were very losely applied and connected to the epidermis. The formation of a distinct ell cluster took place in the early second instar larva. Examination of oenocyte roups treated with osmium tetroxide or with sudan black B (counterstained with carmalum) or examination of freshly dissected groups under phase-ontrast showed conclusively the presence of cytoplasm in the connexions etween the cells and to the epidermis.

In the young second instar larva the oenocytes possessed a dense pale ellowish, finely granulated, eosinophil cytoplasm containing few vacuoles. The chromatic material of the nucleus appeared as a system of interwoven strands and was probably in the polytene condition characteristic of man cells in dipterous larvae. Sparsely distributed granules in the cytoplasm wer coloured purple-black with sudan black and the nucleus and nucleolus wer unstained. The nucleus and nucleolus, however, stained an intense purp with gallocyanin. With 1 per cent. osmium tetroxide and 1 per cent. aqueou eosin the granules stained brown on a pink background. The nuclear mem brane, though spherical in outline, possessed numerous small processes o its outer surface extending into the cytoplasm.

In the late second instar larva the oenocyte nucleus increased in size an became rich in Feulgen staining material. At the same time the number o sudanophil and osmiophil granules greatly increased. The ground cytoplas still stained pink with eosin. The nucleus became asymmetrically situate owing to the enormous increase in the number of granules. When the exuvi space first appeared, the nucleus had decreased slightly in size and the cyto plasm was packed with a very dense accumulation of fine granules tha regularly took up lipophilic colouring agents. The amount of secretion reache a maximum just before the deposition of the epicuticular layers. During th formation of the oenocyte secretory material, the cytoplasm never becam very vacuolated although a few small vacuoles were present. Granules tha coloured with reagents for lipides could be found in the connexions to th epidermal cells. The accumulated secretory material decreased graduall during the deposition of the outer layers of the new cuticle, and the oenocyte showed only faint staining with sudan black B and osmium tetroxide in th newly moulted third instar larva. The shape of the oenocytes did not chang or become lobulated during the moulting cycle as in *Rhodnius* and *Tenebri* (Wigglesworth, 1933, 1947, 1948). The cycle of changes in the oenocytes o *Calliphora* during moulting is shown in fig. 3, A–E.

The lipoid staining material elaborated by the oenocytes differed from tha in the fat body. The dense, finely granulated oenocyte secretion staine diffusely with lipophilic colouring agents within an eosinophil background cytoplasm and not as a series of separate globules or vacuoles. Althoug staining a deep blue or purple to black with sudan black and red with sudan IV similar to the fat-body globules, the oenocyte secretion was stained golde brown to deep brown in 1 per cent. osmium tetroxide and not grey or blac as were the fat-body globules. When the oenocytes were extracted wit acetone and then stained with sudan black a blue-grey tint was still given b the cytoplasm. This staining reaction after acetone extraction is thought t be due to the phospholipide in the cytoplasm. In impregnating lipide wit osmium there are two reactions: (1) an immediate browning reaction calle the 'primary reaction' which is attributed to double bonds, as in oleic acid an similar unsaturated compounds, and (2) a blackening reaction which may o may not follow the browning. The second reaction is poorly understoo (Lison, 1936; Cain, 1950), but is given by saturated fats. The staining of th lipoid in the oenocytes never proceeded to the jet-black stage, and this suggested that the lipoid was of an unsaturated nature.

Numerous histochemical and stain tests were applied to the oenocytes in attempt to determine the nature of the material formed by them, and sults are summarized in Table 2.

ABLE 2. *The reaction of the oenocyte cytoplasm to histochemical tests and stains during the secretory period*

Test	*Observations*
otchkiss polysaccharide	Negative
est's carmine	Negative
old Millon's reagent	Faint pink
inhydrin (0·2 per cent.)	Faint blue
Oopa' reaction for polyphenol oxidase	Negative
Nadi' reaction for cytochrome oxidases	Negative
etrazolium chloride reaction for dehydrogenase activity	Negative
anus green (0·005 per cent. vital)	Few granules green
Methylene blue (0·001 per cent. vital)	No staining
Nile blue (method of Cain)	Light blue (acidic lipoids)
udan black B	Staining deep purple to black
Osmium tetroxide 1 per cent., 24 hours	Brown staining
Baker's acid-haematein test	Some granules pale blue to blue-black removed after pyridine extraction
Schultze's reaction for sterols	Negative
Reduction of acid silver nitrate	Negative
Argentaffin reaction (Lison, 1936)	Negative
Chromaffin reaction (Lison, 1936)	Negative

Paraffin sections stained with Heidenhain's haematoxylin and Mallory's riple stain showed frequently clear spaces in the densely acidophil cytoplasm f the oenocytes (fig. 1, H). Fine fibres ran through these spaces and they are nterpreted as regions where lipoidal material has been dissolved and leached ut of the cells during dehydration and clearing. Oenocytes treated with bsolute alcohol, xylene, or chloroform when they were in the active phase nd then immersed in sudan black, became pale grey. This residual staining s probably due to the 'masked' lipoids that are not removed by normal xtraction procedures. It is also significant that the spaces were only found n cells during the secretory period at moulting and before puparium formaion. Paraffin sections of the oenocytes of early third instar larvae showed no spaces.

The tests indicate that the material formed by the oenocytes contains an acidic, unsaturated lipoid in association with protein. The correspondence of the cycle of increase and decrease in this secretion to the deposition of the epicuticular layers is striking. The staining properties of the oenocyte secretion and the epicuticle are also similar. The oenocyte secretion reaches its maximum development just before the deposition of the epicuticle. Although a transference of material from the oenocytes to the epidermis through cytoplasmic connexions is inferred, it was not possible to trace the incorporation of this material into the epidermal cytoplasm.

It seems highly probable, however, that a function of the oenocytes is the mobilization, elaboration, and transport of a constituent necessary for t formation of the epicuticle. Wigglesworth (1947, 1948), after a critical e amination of the changes in the oenocytes during moulting in *Rhodnius* an *Tenebrio*, suggested that they were concerned with the manufacture of a lip protein which is taken up by the epidermal cells and used in the formati of the 'cuticulin' of the epicuticle.

The secretion of the endocuticle

About 3 hours before ecdysis the surface of the epidermal cells betwee the cytoplasmic processes showed numerous fine vertical fibrils which staine a light blue with Mallory's method. These fibrils united to form a lay which stained blue with Mallory, a pale grey with haematoxylin, and whic gave a weak but positive test for chitin. This layer represents the endocuticl Yalvac (1939) reported in arachnids that chitin was secreted in the form filamentous tufts from the epidermal surface. The chitinous endocuticle w formed around the cytoplasmic processes which extended through it an fanned out like a brush of fine fibres beneath the epicuticle (fig. 1, I). Non chitinous material was added to the epicuticle at the same time as the endo cuticle was deposited. This was also observed by Duarte (1939) in the Africa migratory locust. The microscopic appearance of the outer layers of th epidermal cells during the formation of the endocuticle suggested that it wa formed by both secretion and intracytoplasmic segregation. Weber (1949 stated that the processes of secretion and segregation occur together.

The surface of the epidermal cell at this time formed two types of cuticula material distinguished by their staining properties in Mallory's, haematoxylin and lipophil colouring agents. Epicuticular material was transported by th cytoplasmic processes while endocuticular material is separated between an around them.

The endocuticle was 6–7μ thick at ecdysis and the epicuticle 2μ. Lamella appeared in the outer endocuticle just before ecdysis. The separation of th endocuticle from the epidermis was a continuous process and lamellae wer not detected in it when first secreted. It is probable that lamination an orientation of the chitin-protein substance of the endocuticle takes place afte separation, as suggested by Picken, Pryor, and Swann (1947) for the secretio of the cocoon of *Donacia*. The association between the epicuticle and endocuticle at ecdysis was loose and they separated very readily during sectioning revealing the cytoplasmic processes passing to the epicuticle.

The cuticular spines

The new cuticular spines were formed beneath the spiny region of th cuticle from large folds 7–8μ in height appearing on the epidermal surfac at the same time as the deposition of the outer epicuticular layer. The epidermal cytoplasm extended into these folds, which took on the shape of th spine soon after their formation (fig. 3, F). An acidophil core extended t the tip of the folds, and epicuticle and endocuticle were deposited in a simila nner to the rest of the cuticle. As the endocuticle of the spines was formed, e cytoplasm diminished until there was left only an acidophil strand which tended to the tip of the spine (fig. 1, J) and branched into the epicuticle. e epicuticle was thicker at the tip than at the sides. Two spines developed the new cuticle for one in the second instar cuticle. There was no regular ation between the number of spines formed and the number of epidermal lls. A spine could develop between two epidermal cells.

Before ecdysis the spines when subjected to the 'Nadi' and 'Dopa' reactions lick, 1949) showed the epicuticle at the tip of the spine and the filament

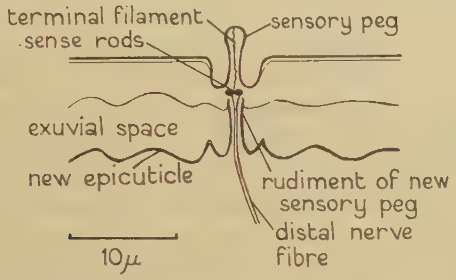

FIG. 4. The formation of a new sensory peg round the distal nerve fibre.

tending from it into the epidermal cell staining blue-violet and deep brown spectively. The reaction was inhibited by hydrogen cyanide and hydrogen lphide. The tests indicated the presence of an oxidase in the inner epi- ticle of the spines. The other epicuticular regions gave a negative reaction this stage. The cuticular spines just before ecdysis also gave a very strong gentaffin reaction and stained a brilliant red in acid fuchsin. These reactions e similar, although localized, to that taking place throughout the epicuticle efore puparium formation as described by Pryor (1940) and Dennell (1947).

he muscle insertions and cuticular sense organs

The muscle insertions and the cuticular specializations of the sense organs e not detached from the old cuticle until just before it is shed. The rudiment the new sensory peg was formed around the axial fibre leading to the ticular peg of the second instar cuticle (fig. 4). The sensory peg stained tensely with haematoxylin and did not contain chitin. At ecdysis a break peared between the newly formed sensory peg and the sensory fibres below e sense rods. The sense rods are shed with the exuvial cuticle. Sihler (1924) served this in the moulting of the sense organs of *Gomphocercus rufus*. It interesting to note that the new sensory peg was formed round the distal erve processes which became after ecdysis the terminal sensory filaments d a new system of axial rods was developed. Their differentiation has not een observed. Only cuticular parts of the sense organs were re-formed at e moult. The number of sense organs did not increase from one stadium the next. The distal sensory processes extended through the endocuticle and epidermis to a group of sensory cells in a cell body immediately benea the basement membrane and were connected to the peripheral sensory nerve

The tonofibrillae are modified non-contractile continuations of the my fibrillae through the cuticle and epidermis. It was observed that the ton fibrillae were not digested by the moulting fluid and were shed with the o cuticle (fig. 1, K). Insect muscle protein contains quite large quantities sulphur and it was thought that the tonofibrillae might also contain protei bound sulphur. The nitroprusside tests (Glick, 1949) and the ferricyani tests (Chevremont and Frederic, 1943) were performed on the cuticle. Th results were unsatisfactory because of the diffusibility of the colour. Howeve the tonofibrillae were the only cuticular regions that stained a diffuse blu Trim (1941) reported small but significant sulphur contents in the larv cuticle of Diptera. The larval cuticle of *Calliphora* contained 0·4 per cent. sulphur. The sulphur content of the third instar cuticle as well as the secon instar exuvial cuticle was determined by the micro-Carius procedure. Th cuticles were thoroughly washed and dried. A sulphur content of 0·35 pe cent. was obtained for the third instar cuticle and 0·32 per cent. for the secon instar exuvium. It is thought that the small sulphur content of the larv and exuvial cuticle is due to the sulphur residues in the protein of the tono fibrillae. The extractable protein (arthropodin) of the larval cuticle contain no sulphur (Trim, 1941).

The break between the tonofibrillae and the new cuticle occurred ju before ecdysis. The tonofibrillae became incorporated in and impregnate with endocuticular material during its deposition and gave a positive chitosa test. Barth (1948) described certain aspects of the moulting of muscle inser tions in *Catacola* (Lepidoptera) and suggested that the tonofibrillae act a supporting fibres ('Stemmfasern') for both integuments during moulting. I is significant that the cuticular sensory system and the muscular (effecto attachments were the last organ systems to break contact with the old cuticl If this were not so, a 'dead period' would occur during the moulting cycl Such a period has never been observed and larvae reacted normally to stimu during the whole period of deposition of the new cuticle.

The cuticle just before ecdysis

When newly moulted third instar larvae and second instar larvae, just befor shedding of the second instar cuticle, were immersed in ammoniacal silve nitrate according to the method used by Wigglesworth (1945, 1948), numerou black spots of silver appeared over the new cuticle (fig. 1, L). The cuticula spines showed a thick deposit of silver extending deep into the cuticle. Th silver deposits in the inter-spinous regions corresponded in number and dis tribution to the filaments extending up the pore canals of the new cuticle Sections treated similarly showed the cytoplasmic extensions to the epicuticl and the epidermal cells as a dark brown or black mass. The outer and inne epicuticular layers at this stage did not reduce ammoniacal silver solution except in the region of the spines. The appearance of droplets on the surfac the cuticle spreading and fusing to form a continuous layer of reducing aterial as described in *Rhodnius* by Wigglesworth (1947) was not found.

The pore canals just before ecdysis contained cytoplasm as far as the inner icuticle. The outer epicuticle separated them from the exuvial space. The ter epicuticle was a homogeneous layer approximately one micron thick, e inner layer had a fibrillar, mosaic appearance and was approximately two icrons thick. This division of the epicuticle is essentially similar to that escribed by Dennell (1947) for the epicuticle of the larva of *Sarcophaga lculata*. The outer epicuticle is sudanophil, osmiophil, stains orange-red y Mallory's method, and is negative to Millon's reagent. The inner epi-uticle also stains with lipophil colouring agents but stains a bright red with cid fuchsin and gives a positive Millon's reaction. Both layers stain black ith iron haematoxylin. Examination of sections with the phase-contrast icroscope showed the outermost epicuticular layer to have a higher refractive dex and to be in a more condensed and presumably more resistant state han that of the inner layer.

No hardening or darkening of the cuticle of the third instar larva took lace except in the cuticular spines and the anterior and posterior spiracles. he reducing spots found in the cuticle are not interpreted as produced by he reduction of the silver solution by polyphenols, except in the cuticular pines where the reduction is very rapid, intense, and localized. The spots vere produced through reduction by the cytoplasm extending up the pore anals and not by polyphenolic material. There was no differentiated exo-uticular layer in the newly moulted cuticle. That the reduction of silver olutions by the cuticle at this stage was not due to polyphenols was further upported by the effects on the cuticle of chloroform extraction and abrasion vith alumina. Newly moulted larvae immersed in chloroform for 10 minutes r rubbed with alumina dust and then subjected to ammoniacal silver solution howed no increase in degree of silver reduction, and in the case of chloro-orm treatment it was diminished.

During the feeding stage of the third instar larva, endocuticle was formed ontinuously and increased in thickness from 6–7μ at ecdysis to 60–70μ at he resting period just before puparium formation. The extensions of the ytoplasm which at ecdysis reaches the inner epicuticle were progressively etracted, leaving behind the typical pore canals visible in fresh sections of the mature cuticle. When growing and feeding third instar larvae were immersed in ammoniacal silver solution, spots appeared on the cuticle surface only at the muscle insertions and cuticular sense organs.

Several larvae that were about to shed their cuticles were cleaned in distilled water with a soft camel-hair brush and dried on filter paper. The exuvial cuticle was carefully removed. The moulting fluid was found in most cases to be completely absorbed and the cuticle surface appeared swollen and shiny. Water droplets pipetted on to the surface showed that it was distinctly, though not strongly, hydrophobe. If the larvae were placed on meat where other larvae had been feeding the cuticle surface became very hydrophil. The wettable, hydrophil nature of the larval cuticle is produce largely from the larval environment. A distinct wax layer or cement laye has not been found.

DISCUSSION

Dennell (1946) demonstrated two layers in the epicuticle of *Sarcophaga* an outer 'lipoid epicuticle' and an inner 'protein epicuticle'. Dennell an Malek (1953) pointed out the resemblance between the inner epicuticle o the *Calliphora* larva and the 'cuticulin' layer of *Rhodnius* (Wigglesworth 1933). The present study shows the development of a two-layered epicuticle The delicate outermost membrane first formed on the outer epidermal cel surface and separating the epidermis from the moulting fluid is regarded a representing the outer epicuticle. Dennell and Malek (1953) have shown tha this layer is highly resistant and contains straight-chain paraffins. The epicuticular material secreted beneath the outer epicuticle from the cytoplasmi processes is regarded as the cuticulin layer and is the only part of the epicuticle of *Calliphora* that corresponds to the epicuticle described by Wigglesworth (1933, 1947, 1948) in *Rhodnius* and *Tenebrio*. Wax layer and cemen layer are absent and the newly formed cuticle of the third instar contains no polyphenol-protein material, or polyphenol oxidase, impregnating the inner epicuticle and subsequently tanning it except in the tips of the cuticular spines. Lipoidal substances are, however, present in both the inner and outer epicuticle at the time of its formation. Dennell and Malek (1953) have shown further that the lipoid in the inner epicuticle is of the steroid type.

Hypotheses on the function of the oenocytes fall into four main groups: (1) excretory function, (2) glandular function, (3) organs of intermediary metabolism, (4) storage organs. They have been described as associated with wax secretion, hormone production, the synthesis of a non-chitinous epicuticular constituent, and recently with the activity of scent glands (Marcus, 1951). Richards (1951) has summarized the literature, and a discussion of the arguments for and against the various theories will not be given here.

That the oenocytes undergo striking changes at moulting was first observed by Verson and Bisson (1891) and subsequently by Stendell (1912), Wigglesworth (1933, 1948), Duarte (1939), and Kramer and Wigglesworth (1950). Willers and Dürken (1916) noticed that the peak in the activity of the oenocytes was reached during the deposition of the cuticle and thought they were associated in some way with chitin formation. Evidence is presented here for the participation of the oenocytes in the elaboration and transfer of a non-chitinous cuticular component, probably lipoprotein or a mixture of protein and lipoid (cf. Wigglesworth, 1933, 1947, 1948). The synchronization of the oenocyte cycle with the deposition of the new epicuticle and the similarity in the staining properties of the oenocyte secretion to that of epicuticular material when it is first formed cannot be overlooked, although an irrefutable proof of the direct participation is extremely difficult. It is not implied that the oenocytes produce the epicuticular material but rather that they are assoted with the production of a precursor substance. The oenocytes according this hypothesis can be regarded as organs of intermediary metabolism in at they are concerned with the translocation of lipoidal material and also glandular organs in that they elaborate and transfer a special necessary bstance to the epidermal cells during cuticle deposition.

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Studies of the Development of the Imaginal Cuticle of *Calliphora erythrocephala*

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With two plates (figs. 1 and 2)

SUMMARY

The development of the imaginal cuticle has been studied with particular emphasis the microtrichia and the pupal moulting fluid.

The microtrichia are formed from acidophil filaments of epidermal cytoplasm which main as the cuticular pore canals after secretion of the endocuticle. Microtrichia at the ise of the bristles are associated with nerves.

The cuticle before emergence consists of a single-layered epicuticle less than 1 μ ick and an endocuticle 3–5 μ thick. The epicuticle and the endocuticle of the sclerites e completely sclerotized after emergence.

The pupal moulting fluid was found to be a clear, salt-free, watery liquid containing otein and lipoid and devoid of proteinases or chitinases. No evidence of dissolution as found in the pupal cuticle. The aqueous part of the moulting fluid is absorbed fore emergence and this may be prevented by the addition of salts.

Evidence is presented for the formation of a denatured, hydrophobic, protein-oid film from the moulting fluid on the surface of the epicuticle after emergence.

Resistance to water loss develops after emergence and is not entirely dependent on ticle darkening and hardening. A film of moulting fluid spread and dried on natural d artificial membranes lowers the rate of water permeation. Such films possibly are erative in regulation of water loss from the imaginal cuticle immediately after nergence. Waxy materials appear on the cuticle surface during the hardening phase. heir possible origin is discussed.

INTRODUCTION

PART from studies of the formation of the bristles (Lees and Waddington, 1942; Lees and Picken, 1945; Schwenk, 1947), the imaginal integument Diptera has received little study. In this paper the development of the aginal cuticle of *Calliphora erythrocephala* Meigen has been studied with rticular reference to the formation of the microtrichia and the properties of e pupal moulting fluid. The histological observations have been restricted ainly to the abdominal cuticle of the imago.

MATERIALS AND METHODS

Pupae of known age were obtained by isolating larvae at the white puparium age from larval cultures and transferring them to an incubator at 24° C. for rther development. Under these conditions the beginning of the true pupal

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period marked by the extrusion of the pupal horns and the appearance of a air space between the prepupal and pupal cuticles, occurred 24–25 hou after the white puparium stage.

Immersion of the puparium in water at 60° C. for 5–10 seconds great facilitated the removal of the pupa from the puparium. The pupae were the fixed in Carnoy-Lebrun or alcoholic Bouin. Peterfi's celloidin-paraff embedding procedure after alcohol dehydration gave good results. The stain ing procedures used were Heidenhain's iron haematoxylin and Mallory triple stain. Details of special procedures are given in the appropriate places the text. Fine tungsten needles prepared by dissolving the metal in fuse sodium nitrite were used for the preparation of peelings of the cuticle fo examination under the electron microscope.

OBSERVATIONS

The Epidermis

The imaginal epidermis of the head and thorax develops from the peripher cells of the imaginal disks, and in the abdomen from two pairs of histoblas located dorsally and ventrally to the dorso-ventral muscles of the body-wa in each abdominal segment except the last of the third instar larva. The histoblasts are present in a quiescent state throughout the larval developme and do not appear a few hours after puparium formation as stated by Bodenstein (1950) for *Drosophila* (fig. 1, A). The epidermis of the genital segment the imago develops from genital disks located in the mid-ventral region of th last larval segment. During the prepupal period (period from the formatio of the puparium to the extrusion of the pupal respiratory horns) the imagin epidermal cells increase in size and number and after the formation of pup cuticle they spread out on the outside of the larval epidermal cells and displac them into the body cavity of the pupa where they are phagocytosed. Th imaginal epidermis is continuous over the abdomen 55–60 hours afte puparium formation. The bristle-forming cells can be clearly distinguished this stage by their large size. The first indication of the formation of the ne imaginal cuticle occurs in the pupa 80 hours after puparium formation. Th

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FIG. 1 (plate). A, group of imaginal histoblast cells within the epidermis of the third inst larva. Romano's silver technique.

B, electron micrograph of the microtrichia 50 hours before emergence. Peeling of abdomin epicuticle. Siemon E. M., kv. 90.

C, electron micrograph of a thin layer of the endocuticle from the abdominal tergum i surface view. The small dark spots represent the pore canals and extend into the microtrichi Siemon E. M., kv. 70.

D, sagittal section of the intersegmental cuticle 24 hours before emergence. The acido phil strands from the epidermis appear as dark lines through the endocuticle, extending int the expanded bases of the microtrichia. Mallory's triple, oil immersion.

E, the reaction of the tormogen cells in the imaginal epidermis to the Nadi reagent 50 hou before emergence. The epidermal cells show no reaction at this stage.

F, drawing of the association between the sensory trichia at the base of the bristle and th sensory nerves.

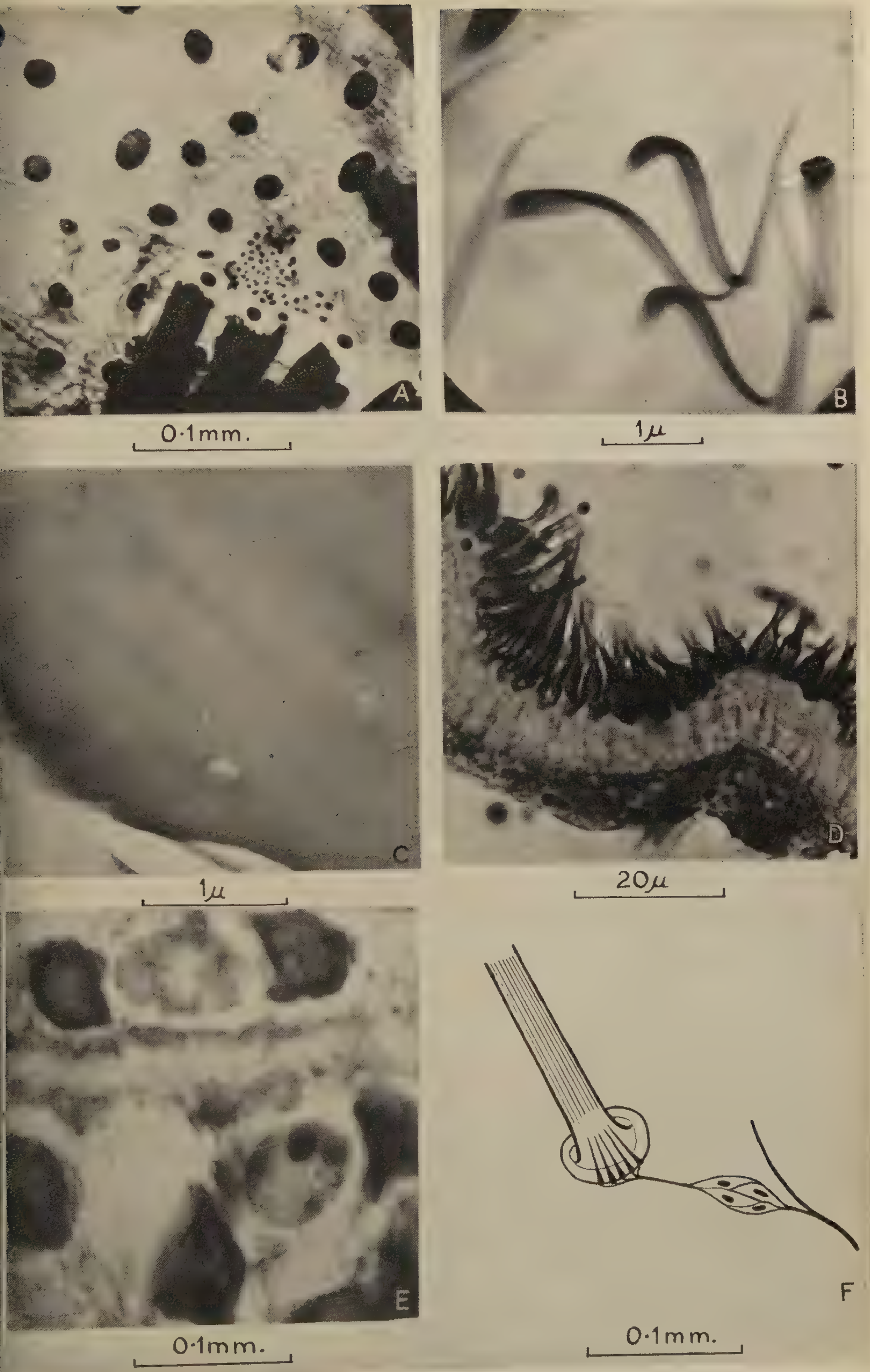

FIG. 1

L. S. WOLFE

idermal cells have contracted away from the pupal cuticle, leaving a fluid-led space between the pupal cuticle and the imaginal epidermis.

he formation of the microtrichia

A delicate layer staining with haematoxylin and acid fuchsin is secreted on e surface of the epidermal cells immediately after the contraction of the idermis away from the pupal cuticle. At this time filamentous extensions are creted by the epidermal cytoplasm. One of these extensions is produced by ch cell and they become the microtrichia of the imaginal cuticle. After their itial formation they do not increase further in length and lie as flexible cell airs against the newly deposited epicuticle (fig. 1, B). The microtrichia on e abdominal terga are 4–5 μ in length.

The cuticle in the 6-day-old puparium shows double staining with Mallory's ethod. An outer layer, the epicuticle, less than 1 μ thick, stains pink and an ner layer, the endocuticle, 2–3 μ thick, stains blue. Fine acidophil filaments xtend from the epidermal cells through the endocuticle and enter the micro-richia. Attempts to colour frozen sections of the imaginal cuticle with sudan lack B were unsuccessful for two reasons; first, the masses of lipoidal material the fluid pupal contents spread over the sections and masked any cuticle taining, and second, the imaginal cuticle at this stage is completely water-oluble. The imaginal cuticle from pupae 2 days before emergence, however, is ot soluble in water and the epicuticle at this time colours with sudan black B. he endocuticle gives a positive chitosan test for chitin whereas the epicuticle nd the microtrichia do not. The microtrichia of the ventral abdominal inter-egmental region possess expanded bases which contain chitin. These are ormed from folds which appear at the time the microtrichia are secreted. ndocuticular material is secreted into these folds but does not extend to the ips of the microtrichia. The chitin-protein complex of the endocuticle is ecreted from the epidermal surface around the acidophil extensions into the microtrichia. The epicuticle does not increase in thickness during the forma-ion of the endocuticle. No further cuticle deposition takes place in the imago fter emergence. The thickness of the cuticle of the abdominal terga is approximately 5 μ; the intersegmental cuticle is 2–3 μ thicker.

Connexions between the epicuticle and the epidermal cells were only found n the microtrichia. Sections of the cuticle after emergence show no signs of ore canals.

Thin peelings of the endocuticle of newly emerged flies with their air sacs filled were examined under the electron microscope and revealed faint regularly distributed dark spots (fig. 1, C) which corresponded in number for a given area to microtrichia. They are interpreted as pore canals and represent filaments of cytoplasm which extend through the endocuticle to the microtrichia. They were clearly revealed as pink strands by staining the cuticle before emergence with Mallory's stain (fig. 1, D). Microtrichia examined under the electron microscope from cuticle removed before emergence showed a well-defined core of dense material. This core was continuous with the pore canals in the endocuticle. The surrounding epicuticle appeared complete homogeneous.

The bristles

The development of the macro- and micro-chaetae and their sockets has bee described in detail for *Drosophila* by Lees and Waddington (1942); Lees an Picken (1945); and Schwenk (1947). Fifty hours before emergence the chaeta have reached their full length and the tormogen and trichogen cells at th stage are reduced in size compared to that during active secretion. Cytoplasm extends into the lumen of the bristle shaft and stains intensely with acid dyes When pieces of the body wall were treated with the Nadi reagent for demonstrating oxidase activity, a very strong positive purple reaction developed i the cytoplasm of the trichogen cells (fig. 1, E). The reaction was inhibited b KCN and by heating to 80° C. and was very much reduced below pH 5. Th purple staining extended up the lumen of the bristles and in pupae 40 hour before emergence even the cuticle gave a positive reaction. The bristles bega to darken between 40 and 50 hours before emergence, first becoming a pal reddish to tan in colour and then changing to a grey which increased in intensity until the bristles were completely black 24 hours before emergence The natural melanization of the bristles occurred simultaneously with th appearance of oxidases in the cytoplasm of the trichogen cell and in the lume of the bristles. The epidermal cells did not contain granules reacting positivel to the Nadi reagent at the same time as the trichogen cells. They did, however, show numerous purple granules 6–12 hours before emergence. Cell underlying the intersegmental cuticle showed no difference in reaction from th cells underlying the segmental cuticle.

At the periphery of the socket of each bristle are located a group of 4– larger microtrichia which are formed like the others from epidermal cells bu are associated here with sensory nerves (fig. 1, F). The function of these receptors is unknown. An association between bristles and nerves has been described by Stern in *Drosophila*. The whole bristle is regarded as a tangoreceptor. From the size and position of these sensory trichia they are more likely to be chemoreceptive.

The pupal moulting fluid

As soon as the outermost layer of the new imaginal cuticle is formed and the epidermal cells have contracted away from beneath the pupal cuticle, the space between the pupal cuticle and the newly developing imaginal cuticle becomes filled with a transparent, watery fluid devoid of cells, called the pupa moulting fluid. Passonneau and Williams (1951) found that a similar pupa fluid from the pupae of *Platysamia cecropia* L. was secreted by the epiderma cells as a transparent gel which did not at first attack the pupal cuticle. However, it later contained proteinases and chitinases that digested all but the sclerotized exocuticle and epicuticle of the pupal cuticle. The fluid was resorbed and replaced by air just before emergence. Isotopically labelled aminoids injected into the fluid were absorbed and incorporated into the proteins the adult moth.

The pupal moulting fluid of *Calliphora* was extracted from puparia after the xth day from puparium formation. A small incision was made through the ipal cuticle between the head and thorax after careful removal of a piece of iparial cuticle from between the respiratory horns. By gently pressing the osterior end of the puparium a drop of moulting fluid exuded through the upal cuticle. By this method, 0·05 ml. of fluid could be extracted from twenty uparia. To obtain some data on its composition, nitrogen determinations were iade by the micro-Kjedahl technique and dry weight and ash determined. he results are shown in Table 1.

TABLE 1. *Composition of the pupal moulting fluid*

	Percentage
Dry weight	5·5 ±0·2
Ash	>0·1
Total nitrogen	0·49±0·02
Protein nitrogen (TCA ppt.)	0·40±0·02
Non-protein nitrogen	0·09±0·04
Protein	2·5

The protein N did not change significantly with age of the pupa.

The fluid reacted positively with all the protein colour tests and also to the alkowski and Liebermann-Burchardt reactions for unsaturated sterols. It ontained no sulphur, phosphorus, or reducing sugars. It reduced ammoniacal ilver nitrate to a dark brown colour and decolorized iodine. Small pieces of ibrin stained in aniline blue, amaranth red, or congo red and soaked in distilled water until the excess dye was leached out were placed in a series of tubes nd 0·01 ml. of pupal moulting fluid added to each with phosphate buffer. The tubes were incubated at 24° C. for 24 hours. No liberation of the dye from he fibrin was observed either in acid or alkaline solution. It is concluded that o proteolytic enzymes are present in the fluid. Small cubes of endocuticle rom larvae were placed in the fluid and when examined after 24 hours they howed no signs of dissolution. Chitinases therefore also appear to be absent. Histological observations revealed no change in the thickness of the pupal uticle during the deposition of the imaginal cuticle. The pupal cuticle was ot sclerotized and consisted of a thin outer lipoid layer and a chitin-protein ayer 2–3 μ thick. Moulting fluid extracted from pupae 24 hours before emergence darkened on exposure to air and blackened on addition of catechol or 3,4 dihydroxyphenylalanine. The reaction was inhibited by heat, KCN, and odium diethyl-dithiocarbamate. This indicated the presence of a polyphenol oxidase. The reaction was not given by the pupal moulting fluid removed from pupae 3 days before emergence.

When the moulting fluid was allowed to dry on a slide, it became viscous, gelated, and hardened to a tough, slightly brown plastic mass. Examination under a binocular microscope revealed sparsely distributed brush-like batch of crystals within the gelated fluid. When the dried fluid was covered by drop of water the protein dissolved, leaving crystals which floated to the surfac The amount of material was too small to perform satisfactory tests on it. is thought that the moulting fluid contains wax-like or lipoidal materials he in solution by the hydrophilic protein of the moulting fluid. The dried flu coloured intensely with sudan black B and was decomposed to a series minute oily droplets on treatment with concentrated chlorinated nitric aci

The gentle bubbling of air from a fine capillary through the fluid form stable bubbles surrounded by a delicate protein film. The bubble surfa dried rapidly and a series of brilliant interference colours were produce After the fluid had been left for several days, the surface-film became wate insoluble and hydrophobic. Formation of a dried thin film of moulting flu leads to the denaturation of the protein and the formation of interferen patterns and a hydrophobic surface.

Water loss through the imaginal cuticle

The surface of the imaginal cuticle is completely wetted by the pup moulting fluid. The fluid will also spread evenly over the extremly hydro phobe outer surface of the pupal cuticle. When flies 12 hours before emer gence, before the moulting fluid is resorbed, were dissected from the pupa cuticle and allowed to dry, the unexpanded cuticle surface still remaine hydrophil and the fly became shrunken and desiccated in a short time. Th surface of the cuticle immediately after emergence, when the air sacs have jus filled, is very hydrophobic and the resistance to desiccation is greatly increase The rates of water loss of emerged and unemerged flies were determined b the method of Wigglesworth (1945) and the results are shown in Table 2.

TABLE 2. *Percentage loss of weight of* Calliphora *imagines after various treatment and exposure for* 4 *hours at* 30° *C. in dry air.* (*The spiracles were occluded as we as possible with Celamel.*)

Treatment	*Loss of weight %*
Fly 12 hours before emergence, rinsed distilled water, dried on filter paper	39·0
Fly 12 hours before emergence, immersed in cold $CHCl_3$ for 3 minutes	60·8
Fly 12 hours before emergence, moulting fluid left on and allowed to dry for 10 minutes in oven at 30° C.	15·5
Fly just emerged, cuticle unexpanded	6·8
Fly just emerged, cuticle expanded but undarkened . .	4·0
Fly 1 day old, cuticle expanded and darkened . . .	3·5
Fly 1 day old, immersed in $CHCl_3$ for 1 minute . . .	64·0
Fly 1 day old, smeared with C09993 (cetyl ether of polyethylene glycol)	61·0

The results show that resistance to desiccation occurs after emergence an that it is not entirely dependent on the darkening and hardening of the cuticle he undarkened expanded cuticle is more resistant to water loss than the expanded cuticle. Chloroform and detergents markedly affect the rate of ater loss.

In order to determine whether a thin film of dried moulting fluid affects ater permeation, experiments were carried out with artificially prepared embranes by Beament's method (1945) (Table 3).

ABLE 3. *The effect of dried films of moulting fluid on the rate of permeation of water through various systems at 20° C.*

System	*Rate of permeation* $mg./cm.^2/hr.$
Water/1 mm. thick gelatin sheet/dry air	15·3
Water/1 mm. thick gelatin sheet/dried film of m.f./dry air	2·6
Water/$CHCl_3$ extracted scale-free *Pieris* wing membrane/dry air	33·7
Water/$CHCl_3$ extracted scale-free *Pieris* wing membrane/dried film of m.f./dry air	1·7

The results show that a dried film of pupal moulting fluid can exercise a ontrol over the water permeation through the extracted *Pieris* wing membrane.

he absorption of the moulting fluid

The imaginal cuticle at emergence is dry. The pupal moulting fluid disppears in the last hour before emergence and is replaced by air. At the time hen the moulting fluid disappears, the volume of haemolymph in the imago ncreases and the ptilinum commences to expand. The ptilinum does not egin to expand unless the moulting fluid has been absorbed. Ligaturing the roboscis before the removal of the moulting fluid did not interfere with the emoval of the fluid. The fluid is absorbed through the cuticle surface. This as shown by Fraenkel (1935), who regarded the unexpanded wing buds as he major cuticular region through which the pupal moulting fluid was aborbed. Fraenkel also found that the wing buds of pupae dissected from the upal cuticle before emergence and immersed in distilled water, became wollen by the uptake of water. This experiment was repeated and confirmed. t was also found that if the flies were immersed in Ringer's solution, the welling did not take place. The addition of 0·002 ml. of a 5 per cent. salt olution, pipetted from a micrometer pipette through a small opening in the upal cuticle into the moulting fluid, prevented the absorption of the fluid y the imaginal cuticle. These experiments indicate that the aqueous part at east of the moulting fluid is probably absorbed through the cuticle surface y osmosis, and that this process is inhibited by hypertonic saline solutions.

In order to observe certain changes taking place during the absorption and immediately before emergence, puparia were fixed to a slide in such a way that normal emergence was prevented and a small window was cut in the puparial wall to the level of the pupal cuticle. The moulting fluid was observed to become progressively more viscous as it decreased in volume, and an a space developed between the pupal and imaginal cuticles. When the fluid w completely removed, the ptilinum began to expand and contract for appro mately 45 minutes, after which the cuticle began to darken. The air sa remained unexpanded. The darkening of the cuticle is, therefore, not depe dent upon the expansion of the air sacs. After the cuticle was melanized, t ptilinum became contracted and disappeared just as occurs in the normall emerged fly. The fly, however, became shrivelled up and died within a fe hours. The cuticle does not darken uniformly when the flies are prevented fro emerging nor does the cuticle surface show the iridescence, silvery lustre, metallic colouring of the fly that has emerged, expanded, and darkened i cuticle naturally. When the inside of the pupal cuticle was examined fro flies prevented from normal emergence and in which the cuticle has darkene numerous black and brown spots were found. These spots were produced b drops of a gelated, tanned, and darkened protein adhering to the inside of th pupal cuticle. The spots lay immediately inside the pupal cuticle and th browning penetrated radially into the pupal cuticle just as if a substance ha diffused from these spots into it. The explanation of the formation of thes spots on the pupal cuticle is not certain but it is thought that they are pro duced by the protein from the pupal moulting fluid gelating between the pupa and unexpanded imaginal cuticle and into which the chromophoric substance which lead to the darkening of the imaginal cuticle have diffused. This observa tion suggested the possibility that the protein part of the moulting fluid wa not absorbed but remained as a film on the surface of the imaginal cuticle forming a thin additional layer. Protein and lipoid has been found in the moult ing fluid up to the time when it is absorbed.

A cuticle layer formed from the moulting fluid

Additional evidence for the appearance of a protein-lipoid layer on th surface of the imaginal cuticle after emergence has been found from electro microscope studies of the epicuticle surface. A comparison of fig. 2, A and B shows that a thin additional layer is present over the microtrichia after th absorption of the moulting fluid and before the darkening of the cuticle. Thi layer, it is thought, is formed from an unabsorbed film of protein and lipoi remaining after the aqueous part of the moulting fluid has been absorbed.

The epicuticle surface examined under transmitted electron beam consistently showed 'shadows' of the microtrichia (fig. 2, C). These are shown only i

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FIG. 2 (plate). A, electron micrograph of the microtrichia 24 hours before emergence. Th core is clearly defined. Siemon E. M., kv. 90.

B, electron micrograph of the microtrichia from the epicuticle of the abdominal tergu immediately after emergence. The outermost layer is regarded as formed from the pupa moulting fluid. Siemon E. M., kv. 90.

C, electron micrograph of the microtrichia from the gena in surface view, showing th 'shadows' on cuticle surface. The shape of the microtrichia is clearly shown. The dark spot between the microtrichia may be cuticular sense organs. Siemon E. M., kv. 70.

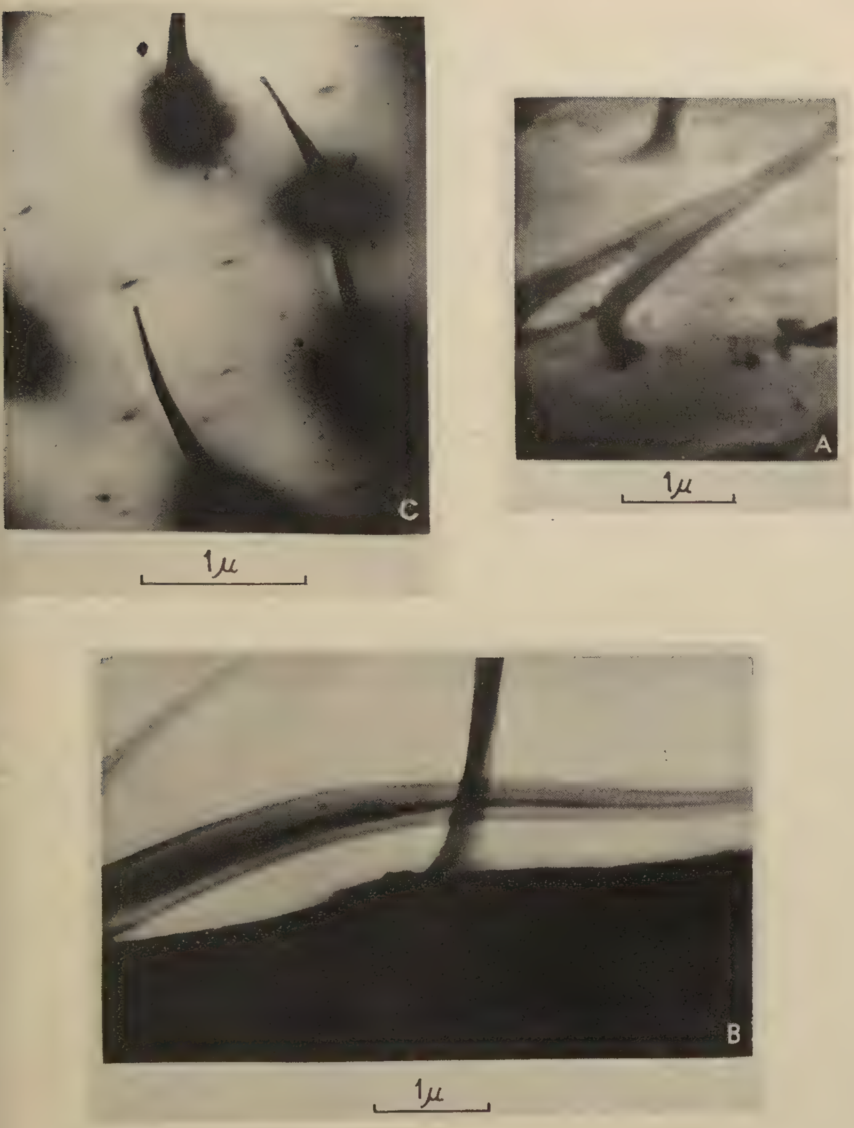

Fig. 2

L. S. WOLFE

eparations of the cuticle from emerged flies with their air sacs filled. They e interpreted as representing thinner regions on the epicuticle surface. The icrotrichia before emergence lie flat against the cuticle surface, but after nergence when the cuticle surface is becoming sclerotized, they lift away om it. If a deposit of protein from the pupal moulting fluid formed a mould und the microtrichia before they lifted from the cuticle surface, the 'shadows' e possibly explained. Examination of several preparations made it clear that e distribution of the 'shadows' was such that it could not have been pro- uced by deflection or scattering of the electron beam by the uplifted icrotrichia.

As the aqueous part of the moulting fluid was absorbed before emergence, e fluid became progressively more concentrated and with the appearance f an air space between the pupal and imaginal cuticles, it gelated. When the y emerged the air sacs were filled, the wings expanded and the cuticle stretched its final fully expanded condition. It is thought that the film of gelated oulting fluid stretches along with the expanding cuticle and forms a delicate urface layer over the epicuticle.

he imaginal cuticle after emergence

Fifteen minutes after emergence, provided the fly has extricated itself from s pupal environment, the air sacs were filled and the thorax and abdomen of e imago became fully expanded. The dorsal cuticle surface showed a silvery ustre as well as greenish interference colours. The wings extended and at rst appeared a whitish opaque colour which changed within 30 minutes to transparent hyalinated membrane, showing a shiny surface and series of idescent colours on both the upper and lower surfaces. Darkening of the uticle began after the first 20 minutes and increased gradually to an intense lack on the thoracic and abdominal sclerites. Sagittal sections of the dorsal bdominal sclerites showed that the darkening began in the epicuticular and uter endocuticular layers. Just before this happened, the entire thickness of he cuticle was stainable by acid fuchsin. No exocuticle was found in the uticle before emergence. In the fully darkened imago the entire endocuticle f the sclerites was sclerotized and melanized. The intersegmental conjunc- iva were not darkened or hardened and the endocuticle remained soft and lexible. The exocuticle, defined as sclerotized cuticle, included both epicuticle nd endocuticle and was completely absent from the intersegmental regions. lthough the sclerites of the thorax and abdomen became completely melanized vithin 45 minutes after emergence, the hardening continued through the irst 24 hours of imaginal life. Darkening and hardening occurred together but hey were not completely interdependent. The hardening could continue lthough the cuticle appeared fully melanized.

Almost all regions of the cuticle showed silvery lustres depending on the lirection of the incident light source. These lustres were produced by light cattering from the rounded tips of the microtrichia spaced one to two microns part. The finer the surface units, the more perfect was the scattering of light

(Mason, 1927). The microtrichia on the genae showed the most perfect lust and it was in this region that the microtrichia were the finest and close together. The microtrichia on the abdominal tergites were arranged in a extremely regular fashion, whereas on the dorsum of the thorax they we irregularly distributed. The long axis of the microtrichia on the abdomin tergites was at right angles to the anterior-posterior axis of the abdome The bases of the microtrichia were directed towards the dorsal median longitudinal axis. There was consequently a narrow median dorsal region whe the microtrichia were not regularly aligned.

The surface of the imaginal cuticle became decidedly more waxy during th first 24 hours after emergence. The origin of the wax has not been determine No specialized wax glands have been found in the epidermis, but this was n specifically studied. A point that may be significant is that the waxy materia appeared on the surface of the cuticle during the hardening phase. It is possib that cuticular, waxy materials are liberated during the orientation and dehydration that occurs as the hardening of the cuticle in the sclerotized region progresses. When 1-day-old flies were immersed for 1 minute in chloroform the interference colours on the abdominal sclerities underwent a slight shi towards the green, suggestive of a removal of a thin layer of material from th surface of the cuticle. The appearance and secretion of waxy materials on th surface of the cuticle of insects is a subject that requires much more intensiv study. The surface of the imaginal cuticle of *Calliphora* is extremely hydrophobic. This is due to the combined effects of the microtrichia and a wax surface.

DISCUSSION

Kroon, Veerkamp, and Loeven (1952), in a study of the process of extensio of the butterfly wing, were of the opinion that the molecular changes observe within the wing during the extension of the cuticle provided an explanatio of the permanent stiffness and hardness of the wing. The orientation of th chitin they regarded as not merely an accompanying phenomenon but a essential factor in hardening. It is significant that although melanization ca be induced in *Calliphora* before the expansion of the air sacs, the cuticle doe not become brittle or waterproof. The physical changes of the protein an chitin fibres upon stretching of the cuticle as the air sacs are expande by muscular activity, appears essential for the process of hardening th cuticle.

The origin of cuticular waxes is still obscure. It is possible that the orientation of the protein and chitin fibres on the extension, dehydration, and sclerotization of the cuticle could lead to the exclusion of some of the weakly boun or labile lipoidal compounds from within the cuticle and to their appearanc on the surface of the 'lipophil' epicuticle and there become oxidized to wax materials. Qualitative observations indicated an increase in waxy materials o the cuticle surface after hardening. Epicuticle wax may arise in the followin ways: (1) as a glandular secretion from the epidermis; (2) by liberation from e cuticle during sclerotization; (3) from lipoid in the pupal moulting fluid. far, special glands secreting wax or a wax precursor have not been observed *Calliphora.*

It is realized that the hypothesis that the protein and lipoid present in the oulting fluid remain as a thin film on the surface of the imaginal cuticle, quires further study and confirmation in other cyclorrhaphous flies. The idence for its existence is based on (1) the failure to demonstrate proteinases chitinases in the fluid; (2) the absence of any sign of dissolution of the isclerotized pupal cuticle; (3) the appearance of a new surface layer on the icrotrichia after emergence; and (4) the appearance of a mould remaining ter the microtrichia lift away from the epicuticle surface. The layer is too iin to be visible in histological sections of the cuticle and in the segmental iticle it is probably sclerotized and melanized. It is thought that the contact ith air of a film of gelated moulting fluid on the cuticle surface would lead to s denaturation and that this would be accelerated by the stretching and rientation occurring when the cuticle became fully expanded.

The cuticle before emergence consists of two layers, an epicuticle (cuticulin iyer) less than one micron in thickness staining with acid fuchsin, haematoxyn, and sudan black and devoid of chitin, and an endocuticle 3–5 μ thick taining with aniline blue and containing chitin. No dermal glands are present nd a cement layer is absent. The endocuticle is perforated by pore canals xtending into each microtrichium. After emergence, the epicuticle and ndocuticle of the sclerites become completely sclerotized and melanized. No ecognizable 'polyphenol' layer is present. Dennell and Malek (1953), in a omparative study of the epicuticle, do not recognize the polyphenol layer as separate layer. In the sclerites of the imago of *Calliphora*, the precursor naterial responsible for hardening and darkening permeates the entire cuticle. Miller (1950) regards the cuticle of *Drosophila* as consisting of only epicuticle nd exocuticle. The exocuticle, however, is formed by sclerotization of epicuicular, as well as endocuticular material, and the sclerites in *Calliphora* and *Drosophila* contain no endocuticle that has not been sclerotized to exocuticle. The arthrodial membranes, however, possess a well defined endocuticle and heir staining reactions are similar to the entire cuticle just before emergence.

I wish to thank Prof. V. B. Wigglesworth, who suggested the research, for is valuable advice and criticism, and I also wish to acknowledge the help and ncouragement given by Dr. J. W. L. Beament and Dr. M. G. M. Pryor. I am indebted to Dr. V. E. Cosslett for the provision of facilities to use the electron microscope.

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On the Relationship between Mammary, Sweat, and Sebaceous Glands

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SUMMARY

. Stages are described in the development of the mammae of male rabbits respond-
to regular injections of oestrogen and progesterone.
. It is suggested that the extra mammary lobes which develop under this treatment
derived from hair follicles and sebaceous glands by the loss of the hairs from the
licles and the proliferation of sebaceous glands and ducts.
. This may throw light on the phylogenetic origin and homology of the mammary
nd.

THE sebaceous glands of the rabbit consist of clusters of about ten cells
almost embedded in the wall of the hair follicles. The cells are large
th an acidophobe cytoplasm and round nuclei. The outermost cells of larger
nds are rather smaller, and possess somewhat flattened nuclei. This
rangement is different from that of the hair follicles where the outermost
lls, corresponding to the deeper layers of the epidermis, have elongated
clei and the inner cells have flattened nuclei; the cells in between have
undish nuclei.

In the nipple of the mammary gland of the male rabbit the sebaceous glands
e about five to ten times the size of those elsewhere. The cells are about
e usual size, and smaller cells with flattened nuclei are arranged around the
tside. Glands of this size cannot be contained within the thickness of the
ll of the hair follicle. Accordingly, they lie outside the follicle and are connected with it by a short duct composed chiefly of cells with rounded nuclei;
, at any rate, there is not a layer of cells having elongated nuclei on the outle of the duct. This duct, then, seems to be formed mainly of cells of the
iddle layers of the rete mucosum of the hair follicle which have burst through
e investing columnar cells of the basal, or outer, layer of the follicular rete
ucosum.

During the course of an endocrinological study, I have had occasion to take
equent biopses of mammary glands of male rabbits which were undergoing
course of regular daily injections of oestrone and progesterone followed by a
teotropin (prolactin or lactotropin) injection. The progressive modifications
the hair follicles of the nipple and their sebaceous glands have proved of
me interest.

uarterly Journal of Microscopical Science, Vol. 95, part 1, pp. 79–83, March 1954.]

The nipple of the male rabbit's mamma is sparsely covered with hair over. At its tip it is perforated by one, two, or three orifices which lead to t mammary ducts. These ducts are simple in section and branch very little the base. There are no alveoli. The wall of the duct is about three cells thi and the cells have large round nuclei. The orifice of the duct is formed by circular depression of the stratified epithelium of the skin of the nipple, whi communicates at its bottom with the duct proper. The communication somewhat eccentric—the duct does not open into the centre of the depressio but slightly to one side; and the depression is filled with a corneal plug, com posed of several layers of half-sloughed stratum corneum. This plug li flush with the general surface of the nipple, and the epithelium of the d pression has a basal layer of columnar cells with elongated nuclei. The nippl like the greater part of the rabbit's skin, is devoid of sweat glands.

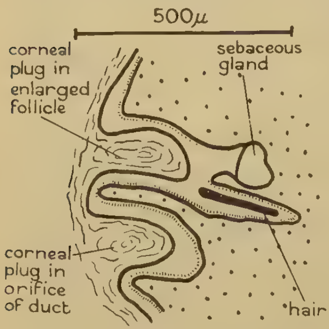
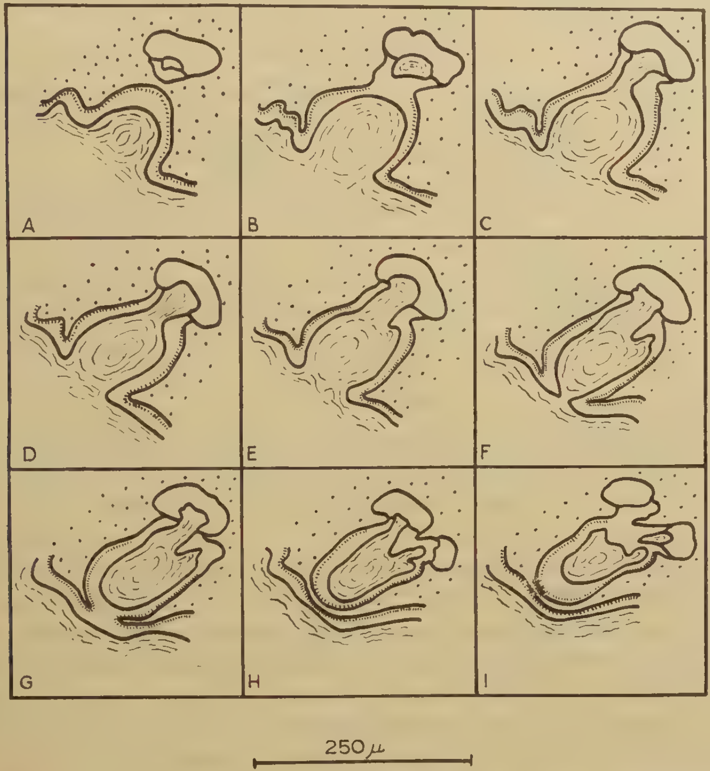

500μ
corneal plug in enlarged follicle
sebaceous gland
corneal plug in orifice of duct
hair

Fig. 1. A camera lucida drawing of a vertical section through an enlarged hair follicle (above) and the orifice of a mammary duct (below). The hair is degenerating and has lost its root. The extent of the epidermal basal layer of columnar cells is indicated by short strokes vertical to the surface.

A short period of regular daily injections of oestrone and progesterone r sults in a proliferation of the duct epithelium and a general enlargement the nipple. The lumen of the ducts becom more capacious and the walls fall into long tudinal folds. The sebaceous glands of th nipple enlarge and many follicles may be see to have several sebaceous glands, or a mult lobed one, each—a condition not seen in th normal male. There is, indeed, more sebaceous tissue present in the nipple of the ma rabbit so treated than in that of the norm male. At the same time the openings of ha follicles near the apex of the nipple enlarg until each hair is lying at the side of a tub several times its diameter. This enlargemen of the lumen occurs only in that part of th follicle distal to the opening of the sebaceou duct; that part below the opening of th duct remains at its original diameter and it completely filled by the hair. The enlarge follicular opening is filled by a corneal plu similar to those on the orifice of the mammary ducts. At this stage the hai root may be seen to be degenerating, with pyknotic nuclei. A little later th hairs in these enlarged follicles are seen to be rootless; they are evidentl being shed. Finally, the hair is shed and little remains of the deeper parts c the follicle, other than a small pouch at one side of the pit which is now a enlarged opening for several sebaceous glands. This pit, however, remain histologically distinct from the proper ducts of the sebaceous glands whic open into it, for its epithelium possesses still a basal layer of columnar cell with elongated nuclei. The sebaceous duct is lined with material similar t that of the corneal plug, but much scantier in amount. The eccentricity of th opening of the sebaceous ducts into the bottom of the follicular pit is marked is a remnant of the lateral position occupied by the glands in the normal icle. It may be noted in passing that sebaceous glands without hairs are l known in the mammae of nulliparous human females (see, for example, ximow and Bloom, 1944, p. 519).

ll these stages may be seen in the first 10 days of a series of daily injecns of oestrone and progesterone. After 25 days of such a series, the proration of mammary ducts is rather more pronounced and some secretory



. 2. Camera lucida drawings of serial vertical sections through an enlarged hair follicle he mammary apex, at a later stage than fig. 1. The hair has been lost and there are now sebaceous glands. A somewhat asymmetric pit is possibly the remnant of the proximal, unenlarged, portion of the hair follicle.

sue has appeared at the lower end of the ducts—alveolar development has gun. But a more pronounced change than this is evident: where before ere were only three mammary ducts at the most, there are now six or eight. amination of serial sections shows that not all of these penetrate as yet to base of the mamma, not all of them are branched, and not all of them ar alveoli. These less developed ducts are evidently new formations. At the tal end of the nipple the ducts cannot be differentiated from each other in croscopical sections. All have the orifice in the form of a depression of the idermis, filled with a corneal plug, and communicating, at one side of its bottom, with the duct proper. In all, the base of the epidermal pit exter slightly deeper at the side opposite the opening of the duct. The duct pro is lined with a definite, though sparse, stratum corneum; it is clearly an e dermal invagination, though devoid of the usual basal layer of columnar c present in the stratified epithelium of the skin and of the orificial depressi

The hairless follicular pits with their associated sebaceous glands are m absent from the apex of the nipple but are present a little distance from apex. Even lower still are follicles as yet retaining their hairs.

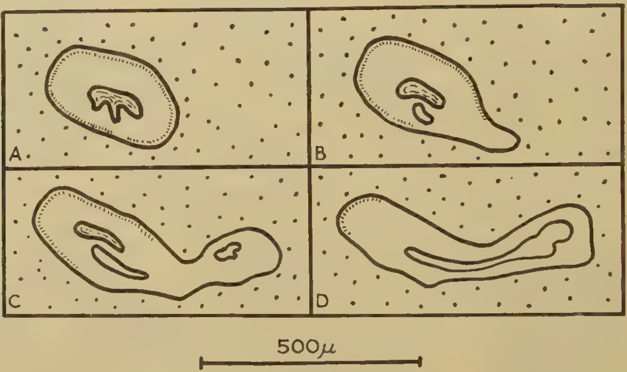


FIG. 3. Camera lucida drawings of almost horizontal serial sections through the region junction of a mammary duct orifice with the duct proper, showing the asymmetry of opening and the continuance of the orificial depression below the level of the opening. T may be a remnant of the proximal part of a hair follicle.

In the later stages of mammary development, induced by injection of s hormones, only one fact is of interest in the present context. The amount sebaceous tissue in the nipple, which at first rose under the influence of t sex hormones, begins to fall again after about the twentieth day, though the is perhaps still a heightened amount around its base. The subsequent c velopment of the ducts and secretory tissue has been too well described Lyons (1937, *a* and *b*) and by others to require repetition here.

It is generally assumed at present that the mammary tissue represents specialized portion of sweat gland tissue (see Maximow and Bloom, 194 The basis for this assumption is the resemblance between the myoepithel or basket cell of the sweat gland and the muscular tissue of the nipple. But t 'basket cells' of the mammary gland are clearly marked off from the glandu cells by a basement membrane running between the two elements (fig. they are unlike the basket cells of the sweat glands which lie inside the bas ment membrane. This has been illustrated many times without mention, figures of sections of mammary glands published in textbooks of histolo (e.g. Maximow and Bloom, 1944, p. 597, fig. 521, where the basket cells appe to lie in the thickness of the basement membrane). The resemblances betwe the musculature of the mammary gland and that of the sweat gland is fficient for an homology to be drawn between the two types of gland on is evidence.

Is it valid, then, to derive the mammary gland from the other chief skin and, the sebaceous gland? The histological evidence that I have before me clines me to believe that it is. I would suggest at in the development which I have described, number of new mammary lobes, that is, indi- dual glands within the mamma, develop in ldition to the extension of those already present. appears that the orifice of each duct represents enlarged hair follicle and the duct proper a baceous duct. There is no evidence to indicate hether or not the secreting tissue is developed om the sebaceous gland, but it seems reasonable presume that this is so, if the other develop- ental sequences described above are correct. his development is fastest near the nipple and roceeds more slowly and incompletely farther own, so that intermediate stages may be found the sides of the nipple. It is interesting to note at a small number of ducts in the nulliparous human female open at the de of the nipple, in the midst of a cluster of sebaceous gland lobes, into pit which is devoid of hair (see Maximow and Bloom, 1944, p. 596, fig. 19). During gestation the sebaceous tissue disappears from these orifices nd they become like those at the apex of the nipple.

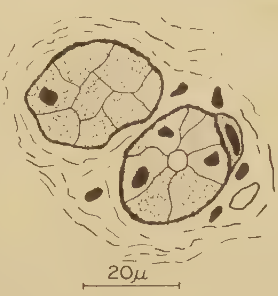


FIG. 4. A camera lucida drawing of two alveoli of the mammary tissue. One alveolus shows the lumen and a 'basket cell'. This cell is outside the basement membrane of the alveolus.

If this interpretation of the origin of the extra mammary lobes and ducts the male rabbit responding to oestrogen and progesterone is correct, then is reasonable to suppose that the origin of all the mammary lobes is from ebaceous glands with their ducts and the associated hair follicle, though oubtless the ontogeny has been abbreviated and they normally develop irectly.

I wish to thank Professor A. C. Hardy and the Director of the Plymouth aboratory for facilities for research, and Mr. A. E. Needham for his help. his work was performed while I was holding a Christopher Welch Biological esearch Scholarship of the University of Oxford and a senior Demyship of agdalen College, Oxford.

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Observations on the Nerve Fibres of *Aurellia aurita*

By ADRIAN HORRIDGE

(*From the Zoological Laboratory, Cambridge*)

With two plates (figs. 3 and 4)

SUMMARY

The nerve fibres of *Aurellia aurita* were originally described by E. A. Shäfer from ld preparations. This work has been repeated with Holmes's method of silver ining on the slide. Results have been obtained substantially in agreement with ose of Shäfer.

The same nerves can be seen in the living animal when a phase-contrast micro-ope or oblique illumination is used. By the use of these techniques, a study has en made of the plan of the nerve net over the subumbrellar surface of the bell.

This work is intended as an anatomical foundation for physiological studies to be scribed elsewhere.

ROMANES (1878) mentions that he saw fibres in the subumbrellar epithelium of living specimens of *Aurellia*, similar to the nerve fibres emonstrated by his friend E. A. Shäfer (1878). The fibres to be described in is paper are of exactly the same kind as the ones that Shäfer described om gold preparations of fixed material. The general appearance, the shape the cell-body, and the position in the epithelium are characteristic, and ere are no other structures with which they can be confused.

MATERIAL AND METHODS

At the beginning of this study a phase-contrast microscope was used for e observation of the living nerves of *Aurellia aurita* Lamarck. The meso-oeal jelly of the exumbrellar surface was cut away to make a flat preparation a w millimetres thick on which the muscular sheet of the subumbrella was isplayed. A 16-mm. objective was sufficient to find the nerves which appeared be right in the subumbrellar epithelium. Later it was found that the nerve ores scatter light considerably, so that they are easily visible by oblique or ark ground illumination. This allowed the use of an ordinary binocular icroscope, as used for dissection, which was very convenient when handling e fibres experimentally.

For the silver-stained preparations whole animals were fixed overnight in a ixture of 3 volumes of saturated picric acid solution in sea-water with 1 olume of 40 per cent. formalin. Strips of epithelium were peeled off from the lly and fastened to microscope slides with cotton or with albumen. Staining as by the method of Holmes (1947) with slight modifications. Well-washed

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slides were soaked in 20 per cent. silver nitrate solution for an hour, th washed again before they were put in the impregnating solution. Impregn tion was for 2–3 days in dilute silver nitrate solution 1 in 10,000, buffered about pH 8 with boric buffer, and with pyridine added to a strength of 1 10,000. I have made use of Dr. Batham's experience with the nerves *Metridium*, but my results have not been so successful as her beautiful pr parations (see the illustrations in Pantin, 1952).

LIVING PREPARATIONS

With a phase-contrast microscope, fibres similar to those described b Shäfer are easily discovered in the epithelium of the concave surface of the be Lower down, in the mesogloea, many supporting fibres are found, but the look quite different, being thin, sharp, straight, and non-cellular. There is n doubt that the fibres seen in the epithelium of the living material correspon to the cellular axons which can be demonstrated by staining. Experiment work (Horridge, 1953) has shown that these living fibres are indeed nerve

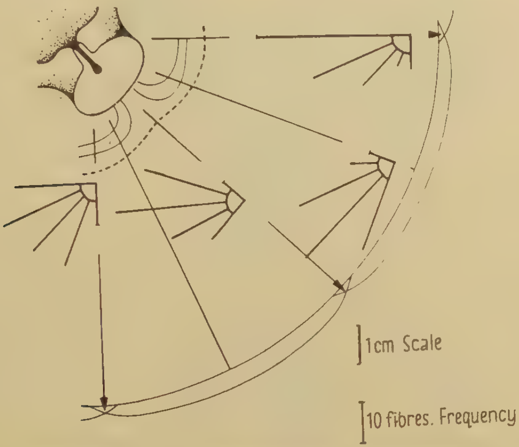
When oblique illumination is used, the great scattering of light makes th nerve fibres appear very wide. Even when allowance is made for this, th axons appear to be full of fluid; some are 12 μ thick, others only 6 μ, mo have a diameter between these two values. In fixed preparations they a collapsed and even ribbon-shaped. The smaller ramifications of these axon have not been seen in studies of living epithelium.

Along the axons are swellings at irregular intervals and although the natu of these is not clear, their appearance suggests that they may be due to damag to the wall of the axon. These swellings increase in frequency under advers conditions. They recall the beading found when nerve axons are vitally staine with methylene blue. Despite the presence of these swellings the single axo in a bridge of tissue between two pieces of *Aurellia* would still conduct th excitation leading to a contraction wave.

Under an ordinary binocular a large living axon can usually be traced fo about 5 mm., sometimes much less. Exceptionally I have been able to follo one for as much as a centimetre. These figures give no idea of the extent o muscle supplied by all the branches of a single neurone, since silver preparations show thin ramifications never visible during life.

In the examination of the living animal with oblique illumination it is ver difficult to make out the cell-bodies belonging to the axons. Fixed preparations show bipolar cell-bodies about midway along the axons; in the livin tissue the observed axons are interrupted somewhere along their length. A this interruption there is an apparent break 10–15 μ long in the course of th fibre and careful examination reveals a large nucleus, a highly refractil nucleolus, and a faint surrounding cell-membrane. It happens that under th conditions of illumination used the cell-body does not scatter light to the sam extent as the axon, but a phase-contrast microscope shows the cell more clearl

At the frequent places where axons cross each other, they appear to do n more than pass one over the other. Since the specimen is viewed from abov h optical conditions that are necessarily inadequate for demonstrating the ailed structure, it is impossible to claim that there is either contact or cytial continuity at these points. However, besides these simple crossings, kinds of more intimate contact, in which the axons twist about each other run alongside, can be found in an exhaustive search; but it is quickly iceable that one sort of relation is the most frequent. Two fibres cross or ne together at an acute angle, run side by side so closely that they are not olved as two fibres in the halo of scattered light, and then they separate and



G. 1. The angular distribution of large nerve fibres at four places on the concave surface of e bell of *Aurellia*. At each place 100 fibres were examined by using an ordinary binocular icroscope and oblique illumination. A histogram with one angular co-ordinate has been drawn at each place; the scale giving the frequencies is shown.

sually resume their former courses. These points where the axons run to- ether correspond to the contacts between neurones seen by other workers Bozler, 1927), and have been referred to as synapses.

Branching of the observed living axons is not unusual and side branches e noticed occasionally, but in general all that are seen are surprisingly raight fibres, which fade out at their ends without giving an impression that ey stop at once.

Taking advantage of this straightness, it is not difficult to plot the angular istribution of the nerve fibres (fig. 1). The principal directions of the observed erve fibres were recorded on a piece of squared paper by the use of a squared yepiece. A small area about 1 centimetre square was scanned systematically, y using a mechanical stage, until 100 fibres had been recorded in the area at ach of four positions on the under surface of the bell, as shown in fig. 1. As ar as possible no fibre was counted twice. The axons run in all directions, ut most of them are sufficiently straight to justify an estimate of the angular istribution. There is no preferred orientation of the nerve fibres except in wo regions of the bell. Along the edge of the bell few of the axons run radially nd in the region just central to each of the marginal bodies the axons appear to radiate from the stalk of the marginal body and spread over the neighbouri epithelium. There is no physiological evidence to suggest that elsewhere the net there is any orientation of conducting pathways. In *Cyanea*, howev there is quite a different organization of the nerve net (fig. 2); the musc fibres are concentrated into distinct blocks. Although the orientation of t nerve cells and processes is quite different, the individual axons are simil to those of *Aurellia*.

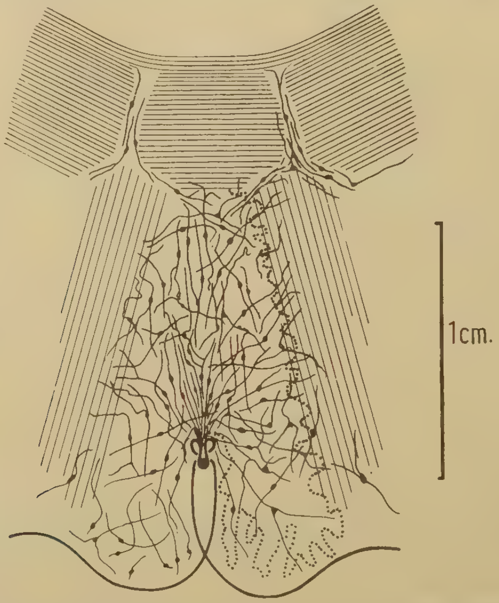


FIG. 2. The large cells of part of the nerve net of *Cyanea* in a muscle-free region. Two the radial muscles and part of the circular muscle are shown. In a small specimen 5 cm. i diameter the number of large nerve cells visible in the most favourable preparations would b approximately as shown.

In *Aurellia* the abundance of the nerve axons seen under an ordinar binocular varies enormously from place to place and between individual Coming from each marginal ganglion is a concentration of axons. Near th marginal ganglia they are often more numerous than elsewhere but not par ticularly so, and this is not true for some specimens. The photograph (fig. 3, is of an area chosen because of the great abundance of axons. In a field of vie 2 mm. in diameter there are usually three or four axons visible and sometime as many as ten, but the number noticed depends upon the conditions illumination, which vary with the thickness of the jelly.

FIXED PREPARATIONS

A few successful preparations were made by Holmes's method of silv staining. The photographs (fig. 4, B and D) show some of the qualities of the nerve fibres in *Aurellia*.

The larger cells, 10–20 μ wide, are not usually stained at all with the silver. ieir large nuclei can be picked out among the much smaller nuclei of the iscle fibres in preparations stained with Heidenhain's haematoxylin. The ost conspicuous feature of the nerve cell is the single, heavily staining, round cleolus in the comparatively large nucleus. A few of the large axons are ore than 8 μ wide. Most are more than 5 μ wide, but towards their ends they t thinner and branch to fine ramifications. The thinner branches stain ore readily with the silver.

These nerve fibres in the fixed preparations show the same relations to ch other as the nerve fibres seen in the living preparations; sometimes two n alongside each other in contact for as much as 50 μ, sometimes they twist gether but always separate again. It seems characteristic of coelenterate rves that they have synapses along the axon lengths, as Pantin (1952) has own. Bozler (1927) depicts, in addition, a different kind of synapse in which very fine axon termination makes a synaptic connexion with the middle of iother axon in *Rhizostoma*, but in *Aurellia* I have not seen junctions of this nd. In *Aurellia* the ends get finer and finer after branching and eventually nnot be traced because they become so very thin. In fact they pass beyond ie resolving power of the microscope. The fineness of the detail excludes any onclusion about the question of syncytial fusion with other thin endings of ie nerve net. However, in the case of the relations between the larger fibres the through-conducting system of *Metridium* (Actinozoa), Pantin (1952) as shown the discontinuity at the synapses between the axons. All the work *Aurellia* (Shäfer; Woollard and Harpman) shows a similar picture. The rincipal dissenter from the view that axons are only in contact is Bethe (1903), nd his figures show fibres continuous from cell to cell, a condition which ther authors deny. It seems likely that Bethe's method of fixation with nitric cid followed by staining with toluidine blue led to artifacts which happened be similar to fibres. Impregnation with heavy metals can produce similar npression of continuity.

The relations, called synapses, between the larger fibres in the nervous ystem of medusae are not as clear as the definite pictures of those in *Metridium*. he axons in *Aurellia* and in *Rhizostoma* come together at some points and un in contiguity for a distance of up to 50 μ, many times their width. The ame can be seen in the living nerve net (fig. 4, A). This is similar to the uccession of small contiguities between parallel fibres described by Pantin. n *Metridium* these large concurrences are found and it is also clear in the ctinian that at the crossing of one fibre over another there is a structure which uggests that excitation can pass from one axon to the other. In medusae these imple crossings have not been demonstrated to be points of contact, although here are indications that such contact exists in my own preparations.

DISCUSSION

The results here described were obtained in a histological study that accom- anied experimental work on the nerve net of *Aurellia* and other Scyphozoa.

By making cuts in different directions, Romanes (1878) showed that th contraction wave is transmitted in all directions across the under surface o the bell. He had evidence that the transmission was through nerve fibres; fo example, in narrow bridges transmission always broke down at a definite poin that could be located with a fine electrode. At Romanes's suggestion, Shäfe (1878) showed that there was in fact a network of nerve fibres which accounte for the physiological results.

Shäfer described a network of bipolar and tripolar cells with processe running in all directions in the epithelium of the lower surface of the bell, ove the circular muscle. He emphasized that, although the fibres run alongside an twist about one another, in no case did he find continuity between the fibre of two cell bodies. In fact he was rather at a loss to explain how the continuit of the contraction wave could be maintained over the whole bell.

The identification of nerve cells in coelenterates has depended upon circumstantial evidence of two kinds (Pantin, 1952): the histological appearance and the agreement of this appearance with the results of physiological experiments. The present paper shows some of the characteristics of the living nerv fibres. The re-discovery that these could be examined has made it possible t show the truth of the inference that these nerve fibres are responsible fo the propagation of the contraction wave (Horridge, 1953). The observation described here give an anatomical background which corresponds with physiological results to be presented in another paper.

A number of authors, particularly Bethe (1903), concluded that the nerv net in several species of Scyphozoa consists of anastomosing fibres which ar continuous from cell to cell. This is contrary to the clear evidence of Bozle (1927) and Pantin (1952), and it seems likely that an appearance of continuit was brought about by artifacts in the preparations.

Bozler (1927) has used vital staining with methylene blue to investigate th structure of the nerve net of *Rhizostoma*. This careful and detailed work ha been largely responsible for the downfall of the belief that continuous fibres run from cell to cell of the coelenterate nervous system. Both bipolar and multipolar cells were described; the former closely resemble the large bipolar nerv cells of *Aurellia* and can be seen without staining. Bozler described two type of synapse between the bipolar cells. There were places where two nerve cell ran close together and then separated again; and there were contacts between the end of one axon and the thicker portion of another. In *Aurellia* only the first of these has been discovered, but Bozler studied a muscle-free area where alternative connexions on to the muscle fibres were not possible.

The results here described for *Aurellia* and *Cyanea* are concerned with the

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FIG. 3. A, the living nerve fibres of *Aurellia* running over the sheet of circular muscle. The photograph was taken through an ordinary binocular microscope with oblique lighting Within the rectangle marked, the apparent gap in the course of the fibre shows the position o the cell-body.

B, the striated fibres of the circular muscle of *Aurellia*, impregnated with silver by Holmes's method.

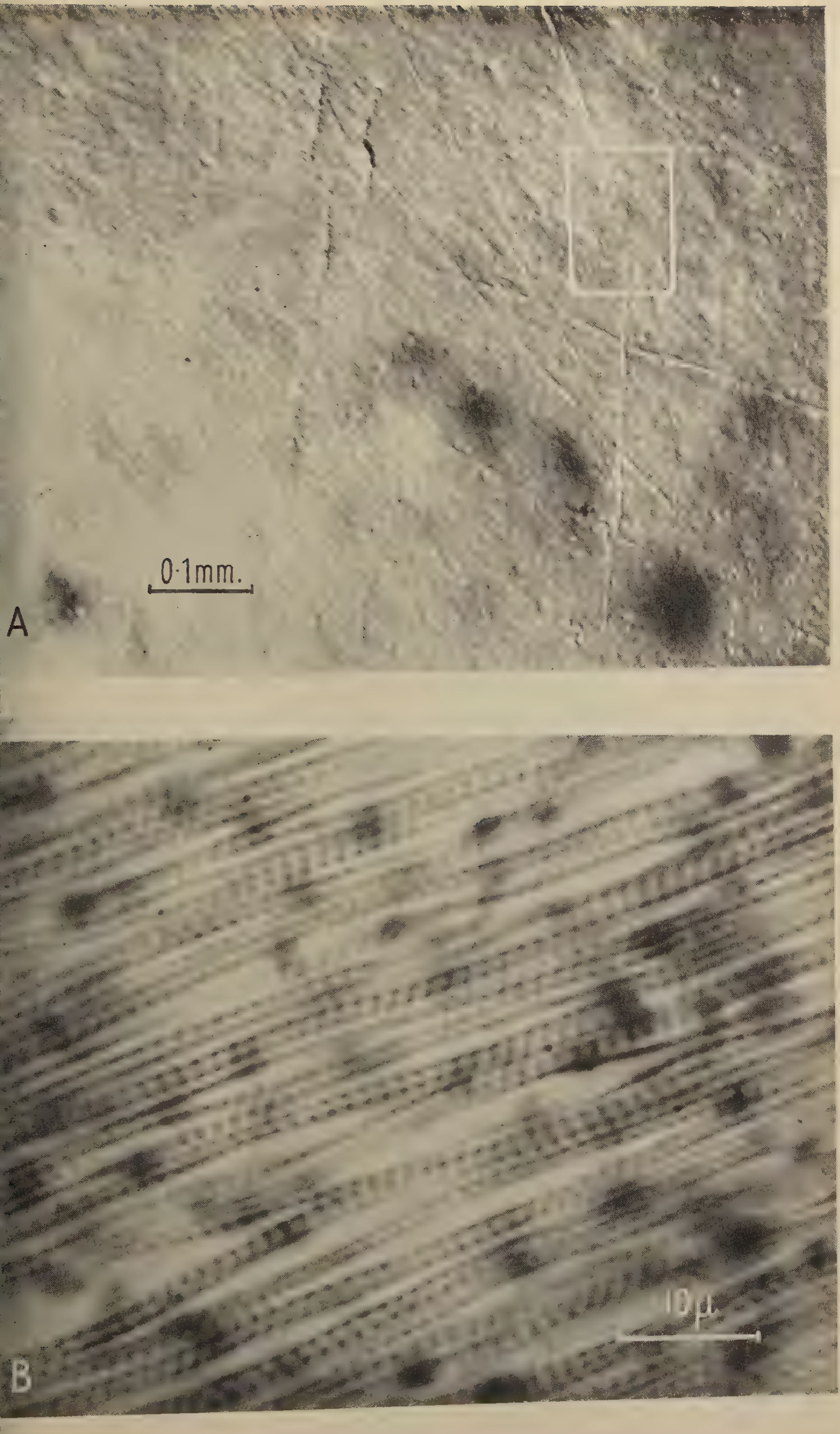

FIG. 3

A. HORRIDGE

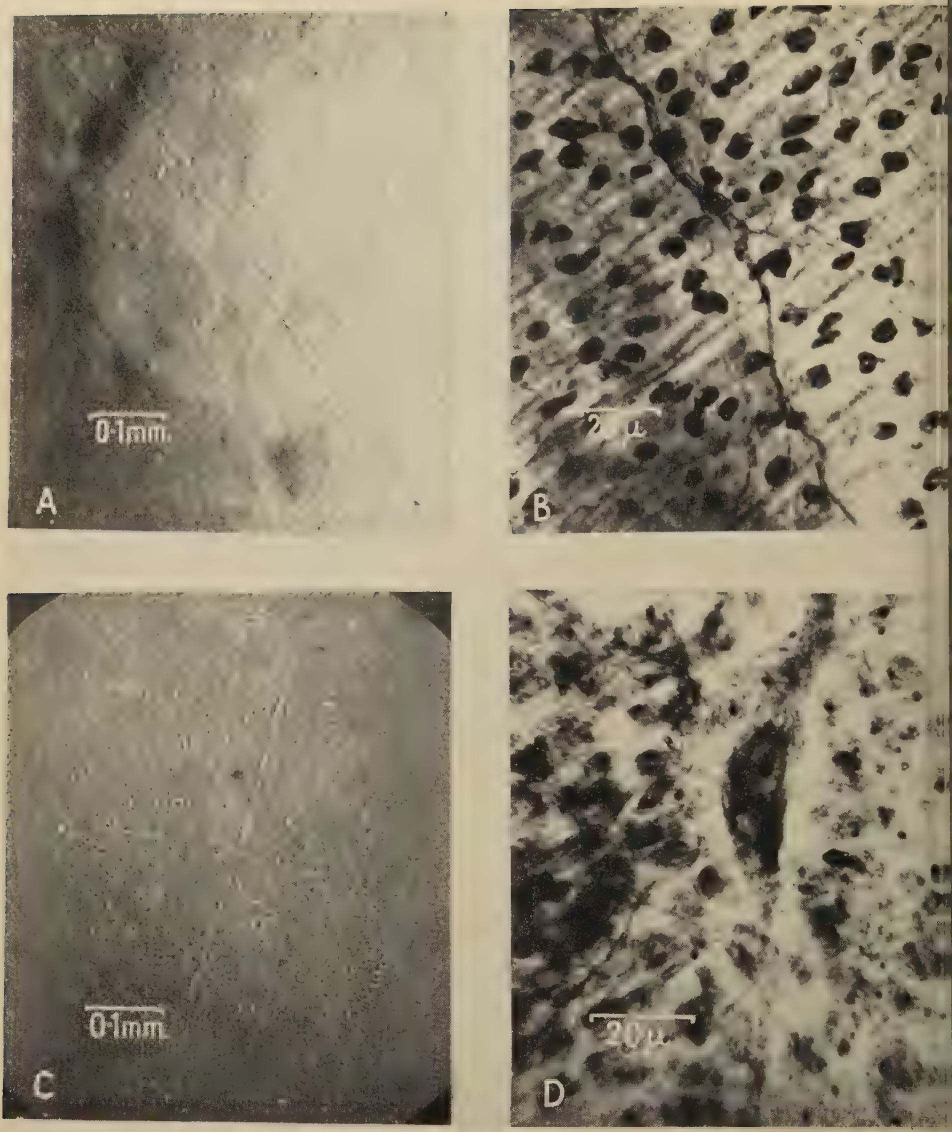

Fig. 4

A. HORRIDGE

ge bipolar cells and their processes which propagate the contraction wave. ie structure of this nerve net agrees with the fibres Shäfer described and details agree with those of the large bipolar cells which Bozler described. iere is general agreement that this network is make up of separate cells iich touch at well-defined points which can be named synapses. This is a stological definition independent of the results of physiological experiments. ie system is normally through-conducting but the existence of synapses ıy become physiologically apparent under the influence of anaesthetics, for ample, magnesium chloride solutions.

Apart from the fact that they are normally through-conducting, these napses differ in certain respects from most synapses in the higher animals ice they appear to occur indiscriminately between any axon of this system d any other. Bozler (1927) suggested that there are several distinct systems large bipolar nerve cells in the Scyphozoa but came to the conclusion that ed preparations would be necessary to be certain of this. The observations on *urellia* indicate that only one system of large bipolar cells is present, and ntacts occur indiscriminately between the fibres of this system. Other nerve lls occur besides those of the through-conducting system and it remains to seen whether the relations between these cells are synaptic contacts.

There is much less agreement about the smaller multipolar cells. Bozler nsiders that axons of all the nervous systems of coelenterates studied are ver continuous from cell to cell and he also draws connexions of a contact ture between multipolar cells and the larger axons of bipolar cells. In *urellia* these multipolar cells were never well stained with silver, and methyne blue produced no results at all. In *Cyanea* there is also a network made up these small nerve cells in places where the large cells of the through-conucting system are absent both histologically and physiologically. In *Cyanea* id *Aurellia* the thinnest fibres of the nerve net are at the optical limit of solution. There is no evidence that the concurrence of a large and a small con, such as Bozler saw, is in fact the site of a connexion, where excitation ay pass from one axon to the other. Where a thinner axon runs alongside a rger the normal forces during growth may be responsible for a misleading ppearance. Much of the difficulty of interpretation has arisen because the nest structures observed in methylene blue preparations of *Cyanea* and lver preparations of *Aurellia* are near the limit of resolution of the microcope.

Nerve fibres have been described by Woollard and Harpman (1939). I uote part of their summary; '2. It is concluded that the nervous system in

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IG. 4. A, 'synapse' between two living nerve fibres. (At the bottom right hand-side there is simple crossing of one fibre over another.)

B, silver-stained nerve fibres running over the muscle fibres among the nuclei of the submbrellar epithelium of *Aurellia*.

C, *Aurellia*, photograph of the living epithelium taken with a phase-contrast microscope show a number of finer nerve fibres besides the two larger nerve fibres.

D, bipolar cell-body in the nerve net of *Aurellia*, silver-stained. The darkly stained nucleolus ppears in the centre of the oval nucleus.

these Coelenterates consists of nerve cells the processes of which are di continuous. 3. The nerve fibres frequently intertwine but never fuse. 4. Nerv fibres terminate on muscle cells by small expansions.' They studied tentacle mainly and were dealing with small fibres of the primary sensory nerves an the connexions of small fibres with the locally controlled muscle fibres. The decided that end to end fusion of axons does not occur but that the smalles axons disappear beyond the limit of resolution.

In his Croonian Lecture, Pantin (1952) has described and illustrated th nerve fibres of the through-conducting system of *Metridium senile*. Both func tionally and histologically these are similar to the fibres of the nerve net c *Aurellia* and it seems likely that a study of one leads to conclusions valid fo the other. In the present description of the axons found in *Aurellia*, compari sons are made with the structures found in the anemone.

This work has been carried out during tenure of a grant from the Depart ment of Scientific and Industrial Research. I would like to thank the Directo and his staff at the Marine Laboratory, Millport, for their facilities an interest, and also Dr. Pantin for encouragement and criticism of the manu script.

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Study of the Embryonic development of the Gooseberry Sawfly, *Pteronidea ribesii*

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om the Hope Department of Entomology and the Department of Zoology and Comparative Anatomy, Oxford)

With one plate (fig. 9)

Summary

The following points in the descriptive embryology of the sawfly appear to be portant especially for comparison with the higher Hymenoptera, e.g. the honey bee.

1. The earlier stages of development (formation of the blastoderm, germinal layers, .) take relatively more time in the honey bee than in the sawfly, while the later ges of development (organogenesis) take more time in the sawfly.

2. Only two well-defined germ layers are formed—the outer (ectoderm) and the ner (mesoderm). Cells proliferate from the mid-ventral area of the germ band and read out to form the inner layer in the sawfly, whereas in the honey bee it forms as middle plate. Localized proliferations at the anterior and the posterior end of the rm band form two cell-clumps; from these differentiate the midgut epithelium, soderm of the procephalic region of the head, muscle layer of the proctodaeum d stomodaeum, and also the germ cells. These are here called the mesendoderm diments. The coelom sacs are not well defined from each other but form a more or s continuous tube as in the honey bee.

3. Nucleoli appear in the cells and the cytoplasm becomes basiphil immediately er the formation of the inner layer. Changes in the basiphilia of cells during velopment are described.

4. The proctodaeum first appears as an invagination in the upturned (dorsal) part the germ band, which is all used up in its formation, and the edges of the ventral rm band rise up to complete the dorsal closure. The Malpighian tubules develop om the blind end of the proctodaeum and are regarded here as ectodermal; in the ney bee they develop before the proctodaeum is formed. In the advanced embryo e midgut cells have big vacuoles in them and throw out pseudopodia into the lumen the gut, and the cells of the salivary gland acquire large lobed nuclei.

5. Agreement is expressed with Snodgrass's view (1938) that the head is composed four segments and a procephalic region. The labrum has a paired origin (in the ney bee it is unpaired in origin); and as in the honey bee the premandibular segent is not developed. The rest of the body is composed of three thoracic and ten dominal segments. Paired appendages appear on the thoracic and the second to venth and tenth abdominal segments (in the honey bee abdominal appendages are t formed).

6. The germ cells arise from the posterior mesendodermal rudiment. In the honey e they appear to be derived from the genital ridges.

7. There are no blastokinetic movements as in the honey bee.

8. The serosa persists till the hatching of the embryo and secretes a cuticle at an rly age. The amnion is not well developed and is transitory; in the honey bee only ne embryonic envelope forms.

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CONTENTS

INTRODUCTION

MATERIAL AND TECHNIQUE

RATE OF DEVELOPMENT AND CHANGES IN EXTERNAL FORM

- Introductory remarks
- Changes in external form

EARLY DEVELOPMENT

- Formation of the blastoderm
- The yolk cells
- Formation of the germ band
- Formation of the inner layer
- Formation of mesendoderm rudiments
- The germ layer controversy
- Embryonic envelopes

DIFFERENTIATION OF TISSUES AND A GENERAL HISTORY OF THE EMBRYO

- The germ cells
- The nerve cells
- Segmentation
- The head segments
- Alimentary system
- Other organs in the trunk
- Changes in the basiphilia of the cells during development

REFERENCES

INTRODUCTION

THERE is an extensive literature on the descriptive embryology insects, but several problems in this domain still remain disputed an unsolved. The controversies relate especially to the formation of germ layer their homology among insects themselves and with other groups of animal the origin of Malpighian tubules, and the head segmentation. It was therefore decided to study the embryology of the sawflies with special reference t these problems. Further, from the point of view of comparative embryolog it was thought that it would be worth while to see how the sawflies compar in their development with the higher Hymenoptera. The development the sawflies has been studied very little in comparison to the aculeate an the parasitic Hymenoptera even though they are the most generalized grou of the order. The only contribution to the subject that could be found was b Graber (1890), who, in his studies of germ bands of insects in general, ha sketched the external features of the embryos of the barberry sawfly, *Hylotom berberides*. However, the very early stages of development (the maturation the egg and its early cleavages) have been studied by several authors and wi be referred to later on.

MATERIAL AND TECHNIQUE

Of the many sawflies tried, the gooseberry sawfly, *Pteronidea ribesii* Scopol was selected for detailed study. In order to obtain the eggs a male and a femal of the sawfly were released in a Petri dish lined with wet filter paper and con ning a gooseberry leaf in it. The dish was kept in an incubator maintained 25° C. The sawfly was allowed to lay the eggs in the incubator for 1 hour. ggs are laid in neat rows along the ribs on the underside of the leaf at tervals of 1 minute. Eggs were then incubated at 25° C. and hatched in 82 ours on the average. The incubation period, however, varies enormously ith the race of the species and the temperature and humidity conditions Iiles, Thomas, and Hey, 1933).

Many different fixatives, including the fluids of Bouin, Gilson, Carnoy, etrunkewitch, have been used by different workers on this material. In the esent study Zenker's fluid was found to be very satisfactory. Smith (1941) commends Zirkle's method (1930) of dehydration for sawfly eggs to prevent ardening of the yolk. Routine dehydration followed by clearing in turpineol as used and it worked quite well. Proper orientation of the eggs is essential a work of this kind and an electrically heated needle as devised by Faberge nd La Cour (1936) was found very helpful.

Serial sections were cut at 8 μ and stained in iron haematoxylin without ounter-staining. Feulgen's method for deoxyribose nucleic acid and Unna's yronin/methyl green mixture (Jordan and Baker) were also tried on the aterial. All the drawings were made with the aid of a camera lucida and even figures (figs. 9, A–G) are photomicrographs. All measurements are from lide preparations.

Rate of Development and Changes in External Form

ntroductory remarks

Under the controlled conditions in which the eggs were allowed to develop t was found that the rate of development of the whole of the stock used was pproximately the same, and therefore the various stages in the development of *P. ribesii* will be described with reference to the age of the embryos. The following table gives the time relations of the various events n development.

Age of the embryo (hours)	*Stage of development*
1–3	Cleavage nuclei migrating to the cortical layer, more numerously in the posterior part of the egg.
4–5	Syncytial blastoderm (fig. 9, A).
6–7	Blastoderm thickens on the ventral side to form the germ band.
8	Cell formation begins in the germ band (fig. 3); eggs collapse during fixation.
11	Shrinkage of germ band; formation of amnion and inner layer (fig. 4, A and B).
14	Shrinkage complete; amnion disappears; inner layer well defined (fig. 4, C); anterior and posterior mesendoderm rudiments also well defined.
15	Differentiation of germ cells and neuroblasts.
17–20	Formation of limb buds, stomodaeum, proctodaeum, and mesoderm tubes.
26	Formation of tracheal invaginations (fig. 6, A).

Age of the embryo (hours)	*Stage of development*
28–30	Germ cells migrate to the ventral side, ganglion cells begin to g arranged into ganglia, formation of salivary gland invaginatio tentorial apodemes (fig. 6, c) and Malpighian tubules (fig. 9, d).
33–36	Dorsal closure of the anterior part of the head up to the mandibul segment (fig. 6, c); germ cells become enclosed into genital ridge antennae move in front of the mouth (fig. 1, b).
43	Head becomes demarcated from the thorax (fig. 2, a) the yolk fro over the head disappears; the posterior end begins to turn on th ventral side.
52	Dorsal closure complete in all parts of the embryo.
67	Appearance of vacuoles in the midgut cells.
73	Pseudopodia seen in the midgut cells.
77	Cuticle appears over the body wall of the embryo.
82	The larva emerges.

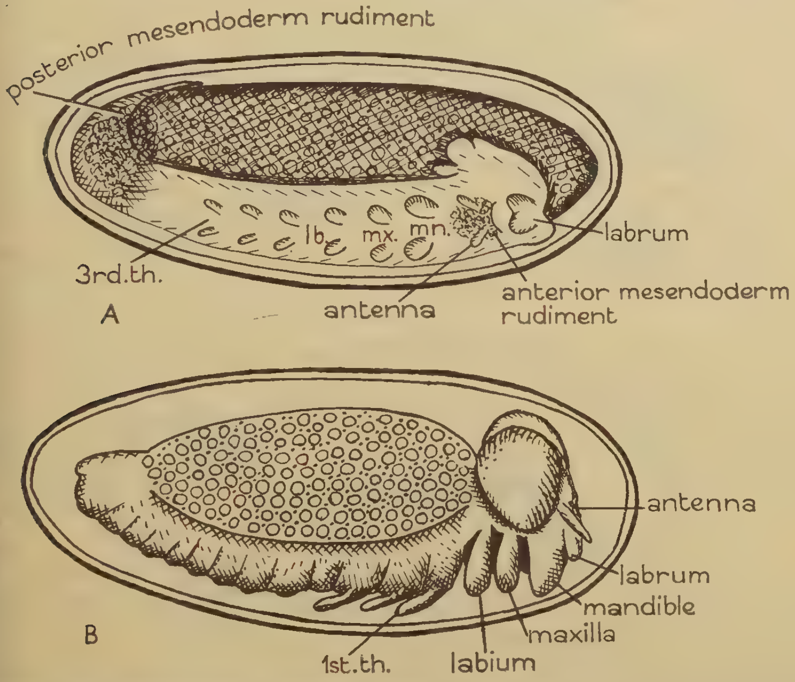
Nelson (1915), following Kowalevski (1871) and Heider (1885), distinguished three periods in the development of the honey bee. They are a follows:

1. The period beginning from the freshly laid egg to the stage when th embryonic envelopes and the germinal layers have been fully formed.
2. The period during which the stomodaeum and proctodaeum form, an head appendages, silk glands, and the tracheal invaginations appear.
3. The period following the above one till hatching.

The honey bee completes its development in 76 hours. The fractions o this time occupied by stages 1, 2, and 3 are respectively six-tenths, one-tenth and three-tenths. The corresponding fractions in the sawfly are two-tenths two-tenths, and six-tenths. Thus the sawfly takes relatively more time for its later development (organogenesis) than the honey bee. In the honey bee, on the other hand, much more time is spent on the earlier stages of development In this respect the sawfly is similar to *Hydrophilus* (Heider, 1885). Nelson (1915) explained this difference in the time relation of *Hydrophilus* and the honey bee by pointing out that the larva of *Hydrophilus* emerges from the egg in a more advanced condition, fitted to lead an active and independent existence which is not the case with the honey bee. The same could be said of the sawfly.

Changes in external form

The external form of embryos can easily be made out in entire eggs, without removal of their chorions, from the age of 18 hours and onwards. At the age of 18 hours the germ band (fig. 1, a) is closely applied to the yolk all along the ventral side. It is divisible into a broad protocephalic or primary head region and a protocormic or primary trunk region. It does not, however, extend up to the tip at the anterior end, while at the posterior end it goes round the yolk and extends on the dorsal side as well for some distance. Limb buds which begin to develop at this stage are also visible on the head and thoracic ments. By the age of 36 hours (fig. 1, B) considerable growth has taken ce especially at the anterior and posterior ends. The head is terminal v, and the head appendages have also moved anteriorly; in the posterior t the upturned germ band develops into the proctodaeum and the edges the ventral germ band rise up to complete the dorsal closure so that this minal end has its definitive shape now. The yolk now lies between the



G. 1. A, embryo at the age of 18 hours. The germ band does not extend up to the tip at the erior end, the yolk forming a cap over it. At the posterior end the germ band turns to the rsal side. In the curved part lies the posterior mesendoderm rudiment; and the proctodaeum pears in the upturned part. Limb-buds have appeared in the thoracic and head regions, in latter is also seen the stomodaeum and anterior mesendoderm rudiment. B, embryo at age of 36 hours. The yolk does not cap over the head now; mouth parts have moved anteriorly, the antennae becoming pre-oral.

lb., labium; mn., mandible; mx., maxilla; 1st. th., 3rd. th., thoracic appendages.

terior and posterior ends of the embryo. Segments are also well demarcated this age. Besides the head segments, three thoracic and ten abdominal gments can be counted. During further growth the posterior end turns to e ventral side as seen at the age of 46 hours (fig. 2, A). Except for these anges of form due to growth there are no movements comparable to blastonesis of many insect embryos. In the honey bee also there are no blastonetic movements. The head by now has attained its definitive form. The st of the body is also well advanced, and its appendages have become ominent. They are present on second to seventh and tenth abdominal segents. Graber (1890), however, in the sawfly *Hylotoma*, has described twelve

pairs of abdominal appendages. In the honey bee, on the other hand, Nels
(1915) did not find any abdominal appendages, though they have been r
ported in other Hymenoptera, e.g. *Chalicodoma*. At the age of 82 hours wh
the embryo is about to hatch, its general appearance is as in fig. 2, B.

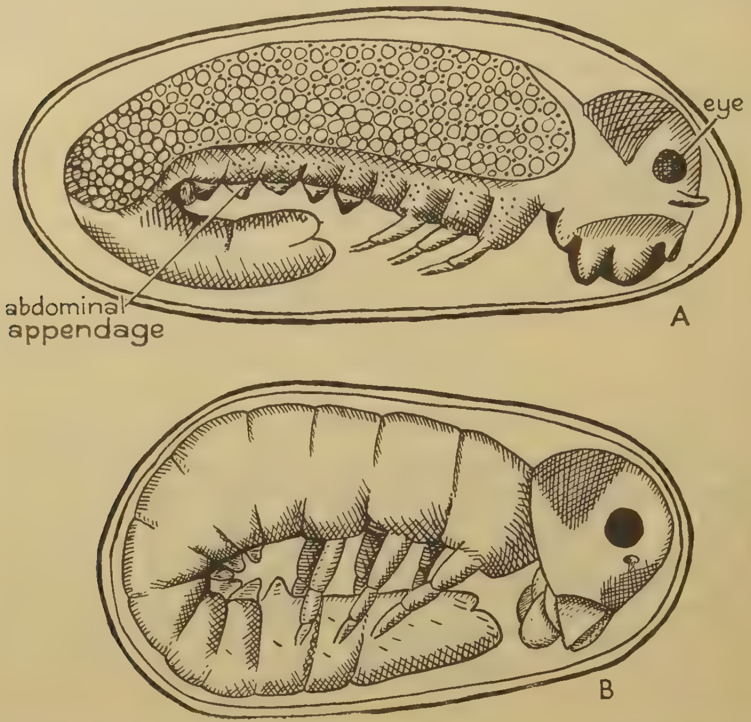


Fig. 2. A, embryo at the age of 46 hours. The head has consolidated attaining almost i
definitive form; the two labial appendages have fused; and posterior end turns on the ventr
side. B, embryo at the age of 82 hours. It is fully formed now and is about to hatch.

EARLY DEVELOPMENT

Formation of the blastoderm

The freshly laid egg is elongated, measuring about 1 mm. in length. It i
invested by two envelopes, the inner vitelline membrane, and the oute
cream-coloured chorion. The chorion is smooth and unsculptured. Th
protoplasm of the egg, as in other insects, is distributed as a thin cortic
layer and as the meshwork which holds together the yolk spheres of variou
sizes.

Doncaster (1904) studied the early development of the unfertilized egg
Pteronidea ribesii. His findings are as follows. In the fresh egg the nucleus
embedded in a little patch of polar protoplasm on the dorsal side at th
anterior end. The first nuclear division is perpendicular to the edge of th
egg. This is immediately followed by another perpendicular division. Thu

r nuclei are produced, lying in a row at right angles to the edge of the egg.
e outer one disappears, the second and third fuse together, while the inner-
st is the egg nucleus which sinks more deeply into the egg. After travelling
ough the yolk for some distance it begins to divide. Sanderson (1932)
ınd that these early cleavage mitoses, in both fertilized and unfertilized
ʒs, are characterized by irregular distribution of chromatin on the spindles,
ormous centrosomes, and large cell-plates. These mitoses were not studied
the present work.

After repeated divisions the cleavage nuclei surrounded by little islands of
otoplasm begin to migrate to the surface of the egg. They become embedded
the cortical layer, thus forming the blastoderm (fig. 9, A). The formation
the blastoderm in the sawfly begins at the age of $2\frac{1}{2}$–3 hours. In the honey
e, on the other hand (which completes its development in 76 hours while
e sawfly takes 82–84 hours), the cleavage cells do not begin migration to
e cortical layer till the age of 8–10 hours. This also shows (as has been
entioned above) that the earlier development of the sawfly is greatly
celerated in comparison to the honey bee.

At the age of 4–5 hours a large number of cleavage nuclei have migrated
the cortical layer and the blastoderm is properly constituted. The nuclei
ach the surface at the anterior and posterior ends of the egg earlier than the
iddle parts of the egg. Further, from the very beginning of migration of
e cleavage cells, more of them appear to migrate to the ventral side than to
e dorsal.

he yolk cells

During the period of migration, while most of the cleavage nuclei reach the
rface of the egg, some of them remain in the yolk to form the so-called
imary yolk cells (figs. 3 and 9, B). It could not be found whether cells from
e embryo also migrate later into the yolk to form the 'secondary yolk cells'
whether the yolk cells are formed in some other way as well, e.g. from super-
ımerary sperms.

ormation of the germ band

When first formed at the age of 4–5 hours, the blastoderm is a syncytium
vering the entire surface of the egg. From the very beginning it is somewhat
icker on the ventral side than on the dorsal. During the next 2 or 3 hours the
ıclei greatly increase in number in the ventral part, which becomes much
icker, forming the germ band. The dorsal part remains thin and constitutes
e serosa (fig. 3, A). The nuclei of the serosa remain in a single layer but the
ıclei in the germ band are irregularly distributed and are more crowded in
e mid-ventral parts than elsewhere. Further, in the posterior end of the
rm band there is a localized thickening in which a large number of nuclei
e present. The nuclei of the germ band are $4{\cdot}3\ \mu$ in diameter. Many of
em do not stain with haematoxylin in the centre and it appears as if they
ad 'holes' in them. Similar 'holes' have been observed in the nuclei of

neuroblasts of the grasshopper, *Cartophaga*, by Carlson (1946), who believ that there is a cytoplasmic core going right through the nucleus.

When the eggs are about 8 hours old they invariably collapse during fix tion. The number of nuclei in the germ band has further increased and it now well differentiated in all parts of the egg. However, it stops short at th anterior end and does not extend up to the tip, so that there is a hood of yol in front of the germ band. At the posterior end, on the other hand, it extend to the tip and rising on the sides of the yolk extends for a short distanc to the dorsal side. In transverse sections, one therefore cuts the germ ban on the dorsal side instead of the serosa (fig. 3, B).

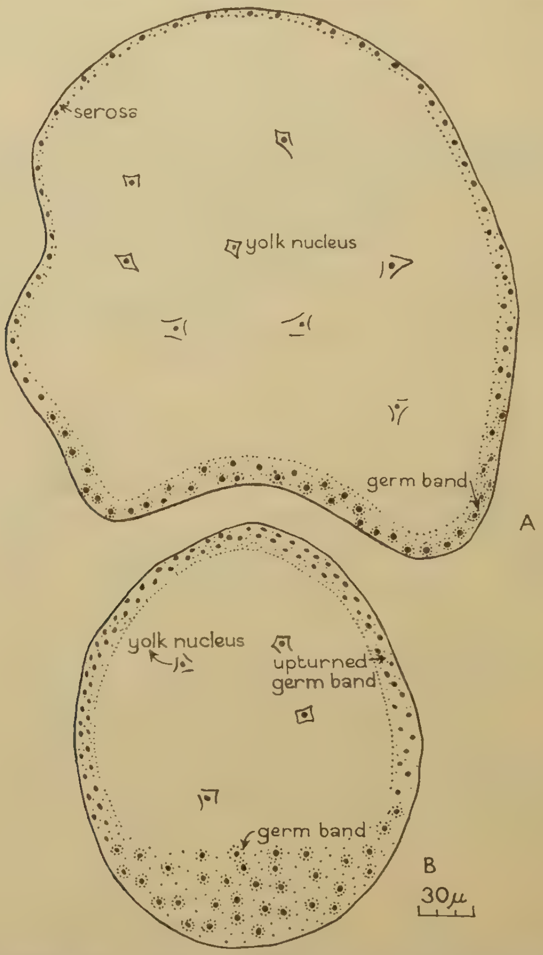


FIG. 3. A, transverse section through the middle part of an egg at the age of 8 hours. Th blastoderm has differentiated into the ventral germ band (showing cell-formation) and dorsa serosa; in the yolk lie the dispersed yolk nuclei. B, transverse section through the posterio region of the same egg as of fig. 3, A. The germ band is broader here, and on the dorsal side th upturned part of the germ band is sectioned instead of the serosa.

Cell-formation also begins in the hitherto syncytial germ band at the age 8 hours (fig. 3). It is especially marked in the mid-ventral parts. Each cleus together with the cytoplasm immediately surrounding it gets separed off as a cell.

In many sections cell-formation is seen to be completed in the anterior and sterior ends of the germ band while the process has not begun as yet in e middle parts, and it appears that the rate of development is higher at the o ends than in the middle part of the germ band.

In some sections of the eggs at this stage, the serosa is seen to break off om the germ band, probably because the germ band begins to shorten. rinkage goes on for the next 5 or 6 hours and reaches its maximum at the e of 13–14 hours.

rmation of the inner layer

At the age of 11 hours the nuclei are seen to be distributed in two or three regular rows all along the germ band and to be especially crowded together the mid-ventral parts (fig. 4, A). Soon afterwards, small cells with rounded ll bodies separate out from the mid-ventral area of the germ band and me to lie more internally (fig. 4, B). These cells spread out, run into each her, and form the inner layer at the age of 14 hours (fig. 4, C). It is possible obtain these stages in the formation of the inner layer in a single egg, as e anterior and posterior regions of the egg develop faster than the middle rts. Thus figs. 4, A and B were drawn from sections of the same egg, B being om a slightly more anterior part than A. From a study of serial sections it pears that the cells separate out only from the mid-ventral area of the rm band. It could not, however, be proved that cells from the more lateral rts of the germ band do not take any part in the formation of the inner yer. Soon after the two layers are formed the cell boundaries in the germ nd become indistinct again (fig. 4, C).

It thus appears that in the sawfly, cells proliferate from the mid-ventral rts of the germ band and spread out to form the inner layer. Satisfactory idence in favour of this view, however, could only be obtained by experiental studies with vital staining methods, &c., and at present it is not ossible to claim definitely that there is no immigration of cells from the surce of the germ band during the formation of the inner layer.

In the honey bee, on the other hand, 'the inner layer forms as a middle ate of the germ band which sinks in and is overgrown by the lateral parts f the germ band'. In some other groups, especially the muscid Diptera, a ell-defined invagination of the median strip occurs which Heider (1885) and thers called the gastrular groove, homologizing it with an elongated blastoore. Henson (1932) also interpreted the anterior and posterior mesenteron udiments of the *Pieris* embryo with the lips of the two halves of blastopore f *Peripatus* and the middle line joining the two rudiments as the closed iddle part of the blastopore of *Peripatus*.

The term blastopore, however, should be applied only in those cases where cells from the surface of the germ band are definitely shown to migra inside to form the inner layer. The routine techniques of serial sections whi have been used in studies of insect embryology are not satisfactory for t study of cell movements.

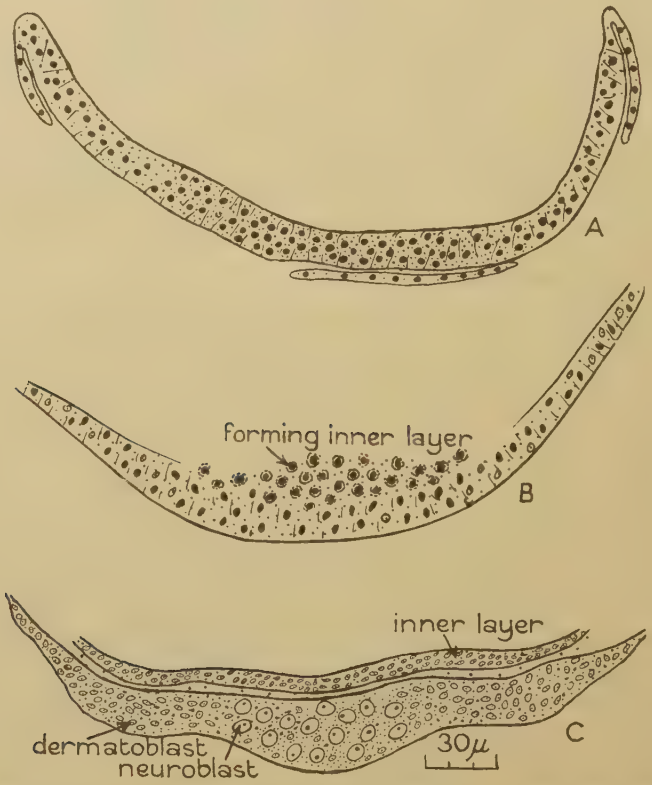


FIG. 4. A, transverse section through the germ band in the middle part of the egg at the age o 11 hours, showing irregularly distributed nuclei, especially crowded in the mid-ventral par Broken parts of the amnion are seen applied to the ventral aspect of the germ band. B, trans verse section through the germ band in a slightly anterior part of the same egg as in fig. 4, A showing the formation of the inner layer. C, transverse section through the middle part of th germ band from an egg 14 hours old, showing dermatoblasts and neuroblasts in the oute layer and the well-formed inner layer.

Formation of mesendoderm rudiments

At the age of 11 hours, when the inner layer is forming, groups of cell become defined at the curved part of the posterior end of the germ band forming a cell-clump. Somewhat later another cell-clump is seen at th anterior end as well, slightly behind the tip. These are here called posterio and anterior mesendoderm rudiments (fig. 1, A) for reasons given later. I this material, as also in the honey bee, it appears that the two rudiment differentiate at the same time as the inner layer. The two mesendoderma rudiments are continuous with the inner layer, which lies between them ey differ from it in that, while the inner layer cells separate off from the er layer as single cells and later on run into each other to form a layer, mesendoderm rudiments are from the very beginning localized pro- rated cell-clumps of the germ band. All the cells of the two rudiments ve the same appearance in haematoxylin preparations.

e germ layer controversy

At present the only purpose of the germ layer concept appears to be for scriptive convenience; and, as Eastham (1930) points out, the controversies st more on differences of interpretation than on concrete results obtained. tensive discussions on the topic are to be found in the literature and so ly a few points will be discussed here.

By common consent the outer layer is called the ectoderm, and the inner ver is generally called the mesoderm. The difficulties are about the homology the endoderm, and there are very various opinions in the literature about e origin of the cells which constitute the midgut epithelium. This is not expected, because, besides any real differences which may exist, the tech- que of serial sections is not reliable for studies of cell-lineage; and other ethods have not been applied to the problem owing to experimental fficulties. From the study of sections of the sawfly material it appears that in is case the midgut epithelium is derived entirely from the two mesendo- rmal rudiments. These also give rise to some muscles and the germ cells, d hence the term 'mesendoderm' used by Henson (1932) is adopted for em here.

In many other insects, cells from other sources also appear to take part in e formation of the midgut epithelium. In this connexion the yolk cells de- rve special mention. Roonwal (1936) regarded the yolk cells as represent- g the first phase of gastrulation, the whole process taking place in several ages. Similarly Eastham (1930) and Johannsen and Butt (1941) and many her workers regard the yolk cells as endodermal in part. The origin and istory of the yolk cells, however, has not been worked out properly. Poly- ermy is regarded as the rule among insects, and it is possible that some of e yolk cells may be derived from supernumerary sperms. Further, it is so not known for certain whether they disintegrate or are incorporated in e midgut epithelium. At present the yolk cells may be regarded (like the mbryonic envelopes) simply as a special feature caused by the large amount yolk in the eggs and probably concerned with the digestion and assimi- tion of the yolk.

mbryonic envelopes

Insect embryos are usually covered by two embryonic membranes—the erosa and amnion. Of the two, the serosa, as has already been seen, is formed y the extra-embryonic blastoderm (fig. 3, A) which breaks off from the germ and during the shrinkage period and gets applied to the vitelline membrane; s two edges grow ventrally and fuse under the germ band. At the age of

18 hours the serosa secretes a cuticular layer, and both the serosa and cuticle persist till hatching (fig. 2, B).

In the sawfly *Hylotoma berberides*, Graber (1890) describes a well-develop amnion that persists till the hatching of the embryo, as in the Lepidopter in the honey bee, on the other hand, only one envelope forms. This develo from the dorsal half of the blastoderm and Nelson (1915) calls it the amnio but goes on to say that it probably corresponds to the serosa of other insec In the sawfly studied, the amnion was found to be a transitory structur persisting for a very short time only. At the age of about 10–11 hours it seen in most parts of the egg. In the sections studied the amnion was nev seen as a continuous layer but in separate parts (fig. 4, A). At the age of hours, when shrinkage is complete, even this rudimentary amnion has entire disappeared (fig. 4, C).

Differentiation of Tissues and a General History of the Embryo

As has already been seen the embryo is very simply constituted at the ag of 14–15 hours, being formed of the two layers—the inner and outer, and t mesendoderm rudiments at each end. This is followed by a period of visib tissue differentiation and growth. The first tissue cells to differentiate are t nerve cells and the germ cells.

The germ cells

Nelsen (1934) has given a general discussion on the origin of germ cells i insects. As he points out, visible differentiation of germ cells has been reported to occur at different stages of development in different insects. I many cases (e.g. *Chironomus* (Ritter, 1890); *Drosophila* (Huettner, 1923)) the are reported to be set aside as 'pole cells' from the early cleavage stages. I others, e.g. the mason bee, Carrier and Burger (1897) describe the germ cel as originating in the visceral wall of mesodermal 'sacs'; in the honey bee als according to Nelson (1915), they are 'apparently derived from the cells constituting the genital ridges'. Nelson says, however, that 'it is not at all unlikely that the germ cells may be set aside at an early period in developmen and afterwards migrate into the visceral wall of the mesodermal tubes, an that such a migration may take place unobserved, on account of the similarit of the sex cells and mesoderm cells'. Further, he suggested that 'if a closel allied form should be found in which the germ cells can be recognized without difficulty, then the behaviour of germ cells in the honey bee could b inferred by analogy'.

In the sawfly studied the germ cells are visibly distinguishable at the age o about 15 hours. They appear as a clump of cells in the dorsal region of th posterior mesendoderm rudiment (fig. 9, B). They are comparatively larg cells, their nuclei measuring 6·4 μ in diameter. In embryos at the age of 2 hours the germ cell-clump has divided into two parts which move backward into the elongated posterior end, passing down laterally. They come to lie o the ventral side as two cell groups at the age of 31 hours. Two hours later th

m cells have become embedded in the dorsal part of the mesoderm tubes
each side. These portions then separate from the rest of the mesoderm,
istituting the genital ridges at the age of 36 hours (fig. 9, C). The genital
ges develop into the sex organs of the larva.

e nerve cells

The nervous tissue in the sawfly develops essentially in the way typical for
ects. Excellent accounts of the process are to be found in the literature
l so a detailed description is not given here.

At the age of about 15 hours some large cells are to be seen in the outer
er of the embryo in the mid-ventral area (fig. 4, C), and also in the dorso-
eral parts of the protocephalic region. These are the neuroblasts, and the
tire nervous tissue will develop from them. The nuclei of the neuroblasts
easure 6·2 μ in diameter. The rest of the cells of the outer layer are smaller,
eir nuclei measuring 3 μ in diameter. The smaller cells are called the der-
atoblasts, as they give rise to the hypodermis and associated structures.
hour or two later, a groove—the neural groove—appears in the mid-
ntral line of the outer layer, and the neuroblasts get arranged on each side
it (fig. 5, B and C), forming the primitive swellings or 'neural ridges'. The
uroblasts last till the age of about 30 hours, during which period they pro-
ice rows of ganglion cells by repeated unequal mitoses (fig. 6, A). In the
ain region, however, the ganglion cells were not seen to be arranged in
gular rows. This is also true of the brain region of the honey bee, where the
uroblasts and their progeny appear to form a confused mass. The ganglion
lls divide again in the honey bee (Nelson, 1915) and some other groups
rosophila, &c.). Some workers, however, believe that they do not divide
it simply grow to become the neurones of the adult. No divisions of the
nglion cells were observed in the sawfly, but they might have been missed.

The neuroblasts in the sawfly, as also in the honey bee and *Pieris*, are not
gmental in origin, and it is only when the segmentation of the body is well
lvanced (at the age of about 31 hours) that the ganglion cells begin to be-
me arranged in segmental ganglia. Nerve fibres begin to develop at the age
about 36 hours in the thoracic ganglia (fig. 6, B) and soon afterwards they
parate off from the ectoderm. In the head segments these events take place
mewhat earlier.

The nerve cells thus acquire their definitive form fairly early in ontogeny,
d it is therefore proposed to study the differentiation and growth of these
lls by cytological methods in some convenient insect material.

egmentation

Soon after the differentiation of the germ cells and neuroblasts, traces of
aired limb-buds begin to appear along the ventral aspect of the germ band.
he inner layer (mesoderm) is at this stage an unsegmented continuous sheet
f cells, 2–3 cells thick (fig, 4, C). At the age of 17–18 hours the free lateral
dges of the inner layer fold over, enclosing a minute cavity. The tubes so

formed are the 'mesodermal tubes' of Nelson and are continuous all throu the embryo (fig. 5, c). This is very similar to the honey bee. It appears th the lumen of the tube is less wide in the inter-segmental than in the segmen

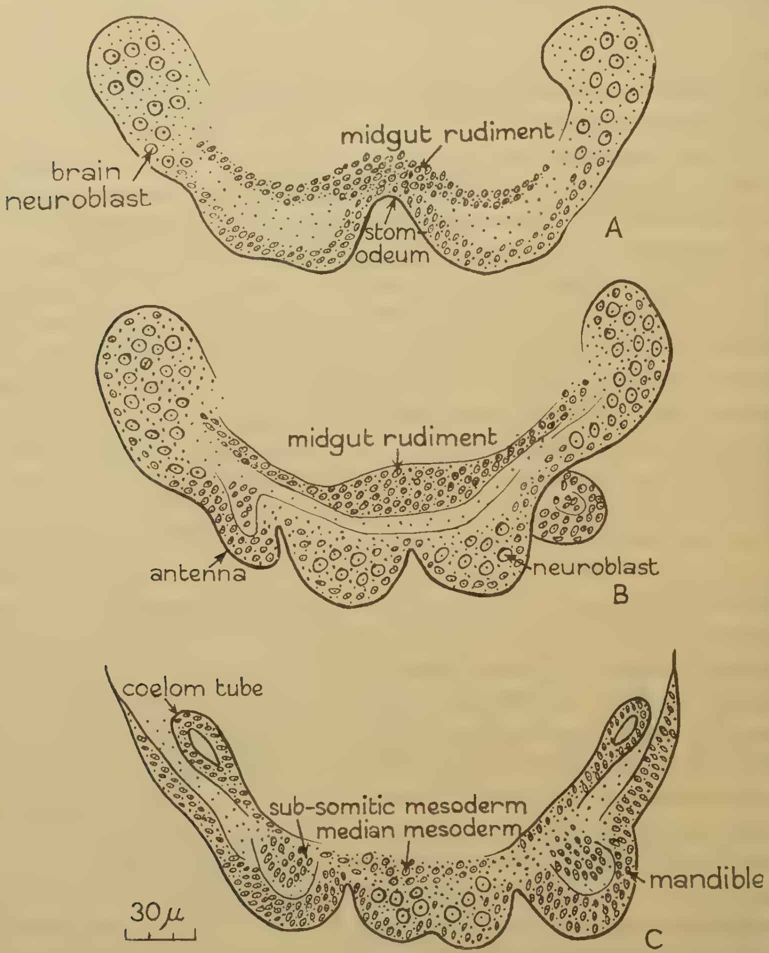


FIG. 5. A, transverse section through the stomodaeal region of an embryo 18 hours old; brai neuroblasts are seen in the dorsal part. B, transverse section through the antennal region of a embryo 24 hours old; the antennal mesoderm is continuous with the mesendodermal rudimen C, transverse section through the mandibular region of an embryo 24 hours old. The mesoder can be divided into median, subsomitic, and somitic (coelomic tube) portions; there ar neuroblasts on either side of the feebly developed neural groove, constituting the neura ridge.

regions. The mesodermal sheet can now be divided, more or less, into thre parts (see Eastham, 1927, on *Pieris*)—the median portion lying immediatel above the neural groove, the somitic part consisting of the mesodermal tubes and the sub-somitic mesoderm, i.e. the part that lies between the median an somitic portions (fig. 5 c). As the limb-lobes develop, a large number of cell n the sub-somitic mesoderm pass down into them. Segmentation of the ecto-m and the appearance of limb-rudiments are the first signs of metamerism remain the most prominent feature in the sawfly. The segmentation of nervous tissue, as already mentioned, begins later. The mesoderm is not rkedly segmented at any stage, and it is difficult to say which of the above tors has a leading role for the division of the germ band into segments.

e head segments

The fully formed germ band, as has already been mentioned, has a broad erior end, the procephalic region, the rest of it being more slender. During contraction period the edges of the broad part rise into the yolk and curl at the anterior end, thus enclosing some yolk between the two arms. At age of about 18 hours, when the limb rudiments are clearly visible in embryo, the head region extends to almost one-third of the whole embryo . 1, A). A description of this region is given below.

Lying in the mid-ventral line of the germ band in the anterior end are two ellings, the labral lobes (fig. 9, E). In the honey bee, however, Nelson re-rts a single labral rudiment from the very beginning. Immediately behind labral lobes and also ventral in position is the developing stomodaeum . 5, A). Most of the mesodermal cells of this region lie in the labral lobes, t some are also distributed irregularly in the whole area. Behind the omodaeum are the developing antennal lobes (fig. 5, B). They are small and ore lateral in position than the labral lobes. The mesoderm in this region is the form of a layer, but it was not seen to form the 'coelom sacs' (meso-rmal tubes). Nelson (1915), however, in the honey bee, reports 'coelom cs' in the antennal 'segment', which differ further from the sawfly in being e-oral in origin. The mesoderm of the antennal region, in the sawfly, is ntinuous with the mesendodermal rudiment, and it is probable that the terior mesoderm at least largely develops from the mesendodermal rudi-ent, as in *Pieris* (Eastham, 1927).

The dorso-lateral wall of the above-described procephalic region of the ead is filled with a large number of nerve cells from which the brain is fferentiated. Lying between the rudiments of the antennae and mandibles e two swellings in the mid-ventral line. Similar swellings were noted in the oney bee by Nelson (1915). They should not be confused with the 'pre-andibular appendages'. They differ from appendage-rudiments in not having mesodermal core: instead they are filled with neurogenic tissue. Actually ey are continuous with the neural ridges of the more posterior part of the nbryo. Further, these ridges continue up to the antennae and appear be-veen them in transverse sections (fig. 5, B). They are probably only the xtreme anterior ends of the neural ridges, as in the honey bee.

Behind is the mandibular region (fig. 5, C). The mandibular lobes develop entro-laterally as evaginations of the ectoderm with a mesodermal core in em. They are bigger than the antennal or labral rudiments. Between the andibular lobes are neural ridges on each side of the median ventral groove.

The mesoderm in this region is a definite layer, consisting of somitic (me dermal tubes), sub-somitic, and median parts, as described earlier. Behi the mandibles are the developing maxillae and labial appendages. Th regions are constituted similarly to the mandibular in regard to mesoder nervous tissue, and appendages, and are comparable to the thoracic segmen

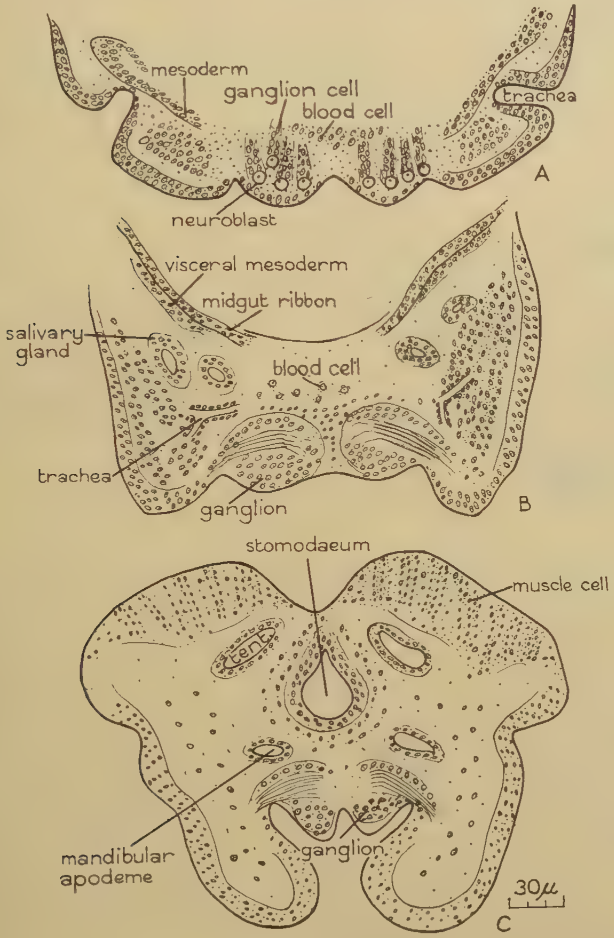
It is obvious that in the sawfly, as in other insects, only mandibular, max lary, and labial regions are 'good' segments, conforming to the strict defi tion of a 'segment'. The controversies about head segmentation relate to t number of segments in the most anterior region. In the present study appears easier to agree with Snodgrass (1938) that the head consists of acron (protocephalic region bearing eyes, labrum, and antennae) to whi four segments—the premandibular (not developed in the sawfly), mandibul maxillary, and labial segments—have been added, than to accept the classic six-segment theory or the theory elaborated by Ferris (1942) and his co leagues. However, it is not intended to generalize on the basis of sawf material, especially because a satisfactory theory about head segmentatio will have to be based on a better understanding of the mechanism the segmentation of the body in general and its modification in the he region.

The further development of the head is concerned with the movements an other changes in the appendages, the approximation of the head segment their growth and dorsal closure, and the development of specialized hea structures.

At the age of 28 hours the labral lobes fuse to form a single unit, the labrum which moves into the front from its earlier ventral position. The antenna also move forward and come to lie in front of the mouth at the age of 36 hou (fig. 1, B). Three or 4 hours later, the mouth parts (maxillae, mandible and labium) have come nearer to each other and are grouped together on th ventral side of the head, and the neuromeres of these segments fuse to for the suboesophageal ganglion. The two labial lobes fuse to form the sing labium at the age of 43 hours. During this period the mesoderm cells, espec ally of the mandibular segment, greatly increase in number so that, at th age of 46 hours, the whole of the posterior dorsal region of the head is fu of them, and muscles are seen to differentiate out of them. While thes changes and growth of the head segments are taking place, the yolk is graduall used up. Thus by the age of 33 hours all the yolk in the anterior part of th head up to the mandibular segment has disappeared and the lateral edge have grown up to meet in the mid-dorsal line, effecting a dorsal closure u to the mandibular segment. Dorsal closure of all the head segments is com pleted at the age of 43 hours and the head becomes demarcated from th thorax.

The salivary glands appear as ectodermal invaginations in the labial seg ment, opening just before the appendage, at the age of 28 hours. They the begin to grow backwards, and 3 hours later have reached the first thoraci segment (fig. 6, B). The cells of the salivary glands are at the beginning lik other ectodermal cells. During later stages they increase in size and uire large lobed nuclei, measuring 10–12 μ in diameter (fig. 7, A).

The skeletal elements of the head also arise as ectodermal invaginations.



G. 6. A, transverse section through the first thoracic segment of an embryo 28 hours old, owing the mesodermal tubes, the neuroblasts in division, and the developing tracheal vaginations. B, transverse section through the first thoracic segment of an embryo 36 hours l, showing the two lateral midgut ribbons with mesoderm layer applied to them, the ood cells in the epineural sinus, and the large number of cells produced from the somitic esoderm (from which will develop the muscles and the fat body). Nerve cells arranged in nglia and forming nerve fibres. Salivary glands growing backwards are also seen in the ction. C, transverse section through the mandibular segment of an embryo 31 hours old, owing the first tentorial and mandibular apodemes and large number of mesodermal cells in the dorsal region. These will form the muscles of the mandibles.

he mandibular apodemes appear as hollow tubular inpushings in the mandiular segment at the age of 31 hours. At the same time a pair of tentorial odemes arise independently just in front of the mandibles and another pair in the inter-segmental region between the maxilla and the labium (f 6, C). They subsequently fuse to form the tentorium. In this the sawfly very similar to the honey bee and *Pieris*.

Alimentary system

The rudiments of the alimentary system appear (as already noted) at t age of 11 hours in the form of the anterior and posterior mesendoderm ru ments. At 18 hours a depression appears on the site of the anterior mesenc derm rudiment. This grows inwards and backwards as a tube, closed at

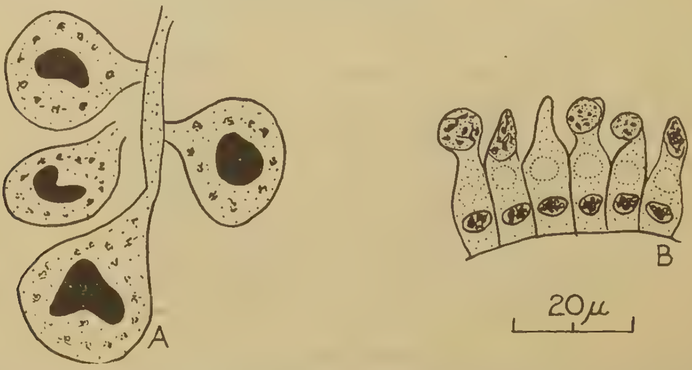
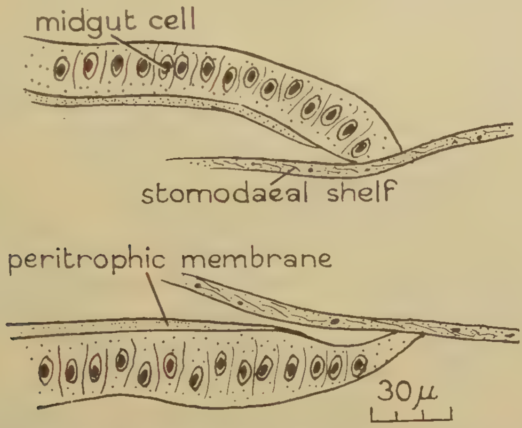


FIG. 7. A, salivary gland cells from sections of embryos 82 hours old showing strongly ba phil and lobed nuclei. B, midgut cells from sections of embryos 73 hours old. The cells ha basiphil nuclei and vacuoles and throw out pseudopodia into the lumen of the gut.

inner end, which pushes the mesendoderm rudiment to a more posteri and internal position (fig. 5, A). The tube is the stomodaeum, and its openin to the exterior is the future mouth of the embryo. While the stomodaeu is being formed at the anterior end, the proctodaeum forms at the hind en It does not develop on the site of the posterior mesendoderm rudiment, bu forms as an invagination in the upturned part of the germ band (fig. 9, B As the invagination develops into the proctodaeal tube, the upturned pa becomes much attenuated and apparently its cells migrate inwards and ar utilized to form the growing proctodaeum. At the age of 36 hours the edge of the germ band from the ventral side rise up to effect the dorsal closure i this region (fig. 9, F). This closure begins at the most posterior end an gradually progresses forwards, giving the definitive shape to the termin region. During further growth, as seen at the age 43 hours, the posterior en turns to the ventral side (fig. 2, A) and grows forwards, reaching almost u to the head when the larva is about to hatch (fig. 2, B).

At the age of 28 hours the blind anterior end of the proctodaeum is throw into loops (fig. 9, D) from which develop the Malpighian tubules. In *Pier* the proctodaeum develops on the site of the mesendoderm rudiment, an Henson (1932) regarded the blind ends of the proctodaeum, and hence th Malpighian tubules, as endodermal. In the sawfly, the proctodaeum develop arately from the mesendoderm and only comes in contact with it later. nson's interpretation cannot, therefore, be applied. The Malpighian ules should better be called ectodermal, in accordance with the origin of proctodaeum. In the honey bee, according to Nelson, the Malpighian ules are formed as ectodermal invaginations which appear before the mation of the proctodaeum. This is further support for the belief that the lpighian tubules are of ectodermal origin.



8. Part of a longitudinal section through the gut from the embryo of an oak sawfly, *Apethymus* sp., showing peritrophic membrane and the proctodaeal shelf.

The cells of the mesendodermal rudiments increase in number and divide to two lateral parts. At the age of about 28 hours, two ribbons of cells ow forwards from the posterior rudiment, closely applied to the yolk on e ventro-lateral aspect. Similar rudiments growing backwards from the terior rudiment develop somewhat later. The two unite, so that at the age 31 hours the yolk on its ventro-lateral aspect has two continuous ribbons plied to it (fig. 6, B). These ribbons spread out over the yolk and cometely enclose it in a thin unicellular envelope. This is the midgut epithelium. may be repeated here that the entire mesendodermal rudiments are not ed up in the formation of the midgut epithelium, the mesoderm of the ocephalic region and that surrounding the stomodaeum and proctodaeum ing also at least partly derived from the two rudiments.

The cells of the epithelium of the alimentary canal undergo several changes efore the hatching of the larva. The hindgut in the earlier stages has a wall rmed of several layers of small cells (fig. 9, F). By the 56th hour the midgut ithelium becomes a single layer of cells (fig, 9, G). The individual cells are ow bigger with more prominent, basiphil nuclei. A similar change occurs in e stomodaeum.

In contrast to the protodaeum and stomodaeum, the midgut epithelium is first almost membranous, being formed of flat cells with nuclei about 3 μ diameter. At the age of 56 hours the cells have grown much in size and the nuclei are now about 5–6 μ in diameter. When the embryo is 67 hours c vacuoles appear in these cells. Six hours later, pseudopodia are thrown o from the inner edges of the midgut cells into the lumen of the gut (fig. 7, The remnants of the yolk are perhaps engulfed. The midgut cells now acqu large basiphil nuclei and granular cytoplasm.

A peritrophic membrane lining the whole lumen of the gut and a stomoda shelf (fig. 8) have been seen in advanced embryos of an oak sawfly, *Apethym* sp. These structures are not prominent in the embryos of *Pteronidea ribe*

Other organs in the trunk

The tracheae appear as a series of segmental invaginations in the late ectoderm of the embryo at the age of 24–28 hours (fig. 6, A). The first p develops on the first thoracic segment and is the biggest of all (fig. 6, A). this connexion it will be recalled that the tracheal invaginations usually d velop on the second thoracic segment and the following nine or ten segmen not on the first thoracic. The occurrence of a tracheal invagination on t first thoracic segment appears to be exceptional. Nelson (1915) found a pa of tracheal invaginations on the second maxillary segment of the honey b (but not on the first thoracic segment). This also appears to be an exception case. When the embryo is about 36 hours old the invaginations have grow into tubes (fig. 6, B). The openings of the tubes on the surface form th spiracles. Cuticular rings develop in the tracheae at the age of about 77 hour At the same time the secretion of the cuticle also begins in the general bod wall, which becomes very much thinned down.

The developmental history of the mesodermal organs is essentially th same as in the honey bee.

The blood cells are produced from the median mesoderm at the age of 2 hours. They are slightly bigger than the other mesodermal cells, their nucl being 4 μ in diameter. Eight hours later, when the yolk withdraws from th developing embryo and leaves a space, the epineural sinus, the blood cell are dispersed into this space (fig. 6, B).

FIG. 9 (plate). A, transverse section through the egg at the age of 3 hours to show the ear blastoderm; the nuclei are strongly basiphil.

B, transverse section through the posterior region of an embryo at the age of 18 hours, showin the proctodaeal slit in the upturned part of the germ band and the germ cells in the anterio most end of the mesendodermal rudiment; the cytoplasm of the cells is now more basiph than the nuclei and there are prominent nucleoli.

C, half of the transverse section through an embryo at the age of 36 hours, showing the genit ridge with germ cells embedded in it which have migrated from their earlier position as i fig. 9, B.

D, transverse section through an embryo 28 hours old showing the beginning of the formatio of Malpighian tubules from the proctodaeum.

E, transverse section through the labral region of an embryo 24 hours old showing the bilobe labrum and the neuroblasts of protocerebrum.

F, transverse section through an embryo at the age of 36 hours showing the multicellul proctodaeum and dorsal closure in this posterior region.

G, transverse section through an embryo 56 hours old showing the heart and the cellul proctodaeum.

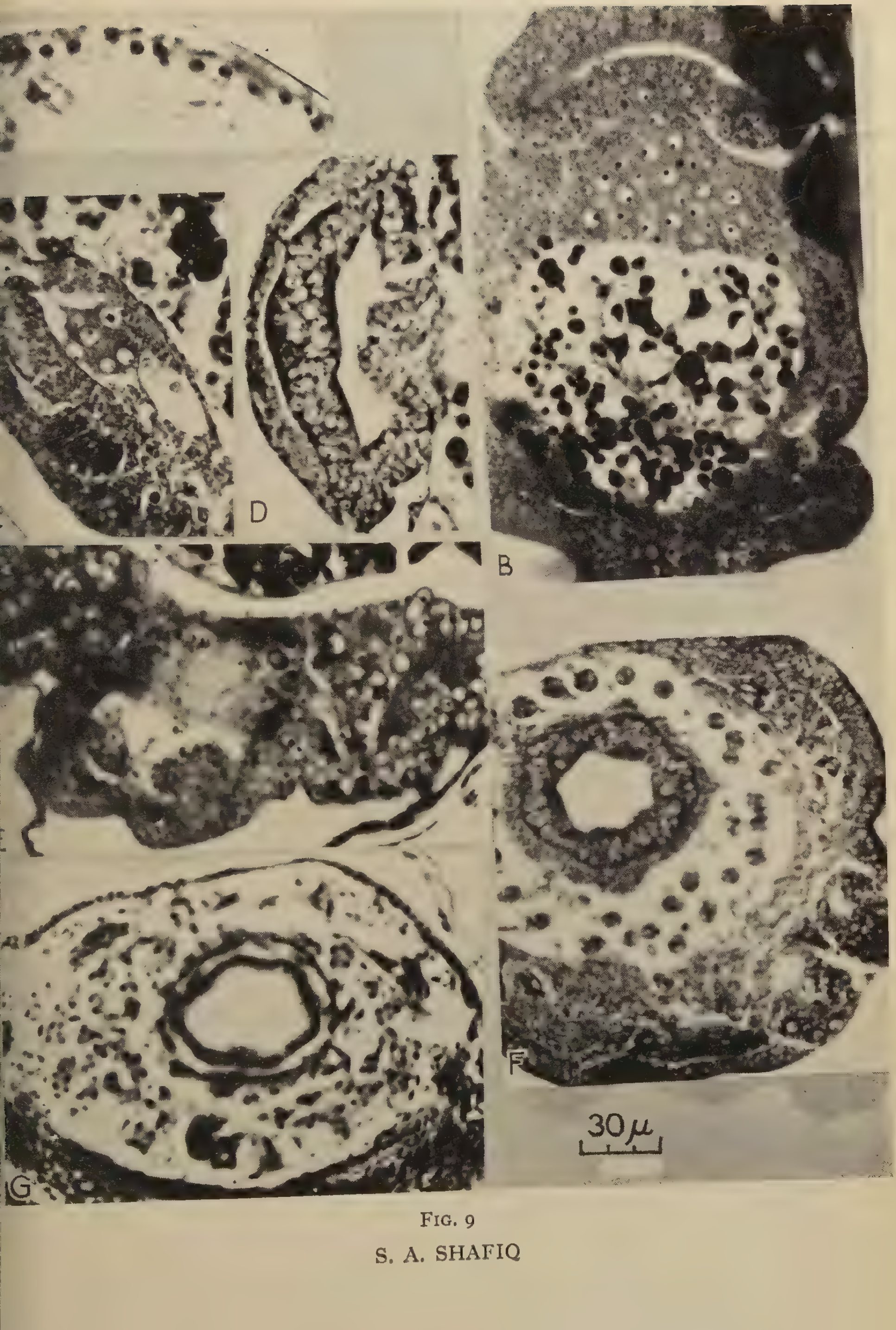


FIG. 9

S. A. SHAFIQ

The sub-somitic mesoderm proliferates into the developing limb-buds of thorax and abdomen and gives rise to their muscles (fig. 6, A).

The mesoderm tubes do not last long as such. At the age of 28 hours they e broken down (fig. 6, A). Their inner walls get applied to the endoderm bons and will eventually form the thin muscular coat of the gut. This is visceral layer. The outer part of the mesoderm tubes adjacent to the body-l is the somatic layer, from which will differentiate the trunk muscles.

The visceral and somatic layers of the mesoderm are continuous in the so-lateral angle. The cardioblasts which usually lie in this region are not ibly different from the rest of the mesodermal cells (unlike most other ects). At the age of 45–46 hours the edges of the embryo rise on the lateral es of the yolk. When the embryo is 52 hours old the rising sides of the bryo have united in the mid-dorsal line and the dorsal closure is completed. e mesoderm is also carried up, and the heart is seen in the mid-dorsal line . 9, G), probably formed by the cardioblasts in the usual way.

anges in the basiphilia of the cells during development

During the course of development changes in the basiphilia of the cells re observed in haematoxylin preparations. Preliminary attempts were de to study the basiphility by the use of Unna's pyronin/methyl green in for differentiating deoxyribose nucleic acid (DNA) from ribose nucleic d (RNA).

In the blastoderm stages (age up to 14 hours) the nuclei stain with haema-xylin and the cytoplasm remains colourless (figs. 4, A and B; 9, A). With ronin/methyl green mixture the nuclei stained with pyronin. Feulgen's thod gave no colour. This would suggest that the nuclei have little highly lymerized DNA.

When the germ layers have formed and the embryo begins a period of sue differentiation and growth (at the age of 15 hours), a prominent change observed in the cells of the embryo. The cytoplasm now stains with ematoxylin and the nucleus has a prominent nucleolus (fig. 9, B). The toplasm and the nucleoli stain strongly with pyronin (probably owing to e presence of RNA), and the nucleus itself stains slightly with methyl een. The nuclei now also give a positive Feulgen's reaction. In this con-xion the work of the following investigators will be recalled. Brachet (1952) owed that in Amphibia the synthesis of RNA and proteins proceed at a gh rate after gastrulation, and Zeuthen (1951) believes that DNA synthesis so parallels that of RNA, there being no synthesis of DNA during segmenta-on. Further, in the study of embryonic nerve cells of mammals, Hyden 943) showed that large amounts of ribose nucleotides appear in the cyto-asm only after the development of nucleoli rich in RNA. The situation in wfly appears to be similar to the above-mentioned instances.

When the embryo is about 31–33 hours old, the hitherto prominent cleoli have largely disappeared, and in haematoxylin preparations the nuclei uld not be stained differentially from the cytoplasm.

In the advanced embryo (age 56 hours) the cells in the various tissues ha developed different characteristics as regards basiphilia (fig. 9, G). Thus t nerve cells have little Feulgen-positive material in them, and the cytopla is strongly coloured by pyronin. The cytoplasm of the cells of midgut, saliva glands, and Malpighian tubules is also basiphil, staining strongly w pyronin. Cytoplasm of the other cells does not appear to be so basiphil.

The work was done under the supervision of Dr. B. M. Hobby, and I ha a deep sense of gratitude to him for guiding me all through the work. It also a pleasant duty to thank Prof. G. C. Varley for accommodating me in t Hope Department, and also for much helpful instruction. I am also grate to Dr. J. R. Baker and Dr. M. Fischberg for kindly reading through t manuscript and suggesting many improvements. Thanks are also due to Pr H. W. Miles and Mr. Gee for supplying the sawflies.

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Growth and Regeneration in the Tracheal System of an Insect, *Rhodnius prolixus* (Hemiptera)

By V. B. WIGGLESWORTH

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With one plate (fig. 10)

SUMMARY

The tracheoles in *Rhodnius* are simple or branched tubes, usually 200–350 μ in ngth, each formed within a single cell, the nucleus of which lies at about 75 μ from e point of origin. They taper gradually from a diameter of 0·7–0·8 μ to end blindly about 0·2 μ.

Once laid down the tracheoles persist unchanged throughout the life of the insect; nlike the tracheae they do not shed their lining cuticle at moulting (cf. Keister on *ciara*). New tracheae and tracheoles arise by the outgrowth of columns of cells from e sides or endings of existing tracheae at the time of moulting.

The tips of the tracheoles migrate actively towards regions of deficient oxygenation, rawing the tracheae after them. Movements up to one millimetre have been observed. his is the mechanism by which implanted organs are provided with a tracheal supply the absence of moulting.

The outgrowth of new tracheae and tracheoles is greatly stimulated in regions of eficient oxygenation. This is the mechanism of 'tracheation' of implanted organs hen moulting takes place.

If the insect is reared in reduced concentrations of oxygen, there is an increase the number of large tracheae developed. This is particularly evident in the wing bes. The general pattern of wing venation is not affected by this altered tracheal rrangement, but minor changes may occur. These are briefly discussed.

IF an organ with a high rate of oxygen consumption is implanted into the abdomen of an insect it becomes richly supplied with tracheae from the ost. That is very obvious when the corpus allatum has been transplanted in *hodnius* and the insect allowed to moult. But some degree of 'tracheation' akes place before moulting occurs; indeed, it is commonly supposed that an nplanted organ will not become functional until its new tracheal supply has een established.

Nothing was known of the histological changes involved in the tracheation f implanted organs. The object of this paper is to describe this process and analyse some of the physiological reactions concerned. But before doing his it will be necessary to describe the normal stages of growth and moulting the tracheal system, about which there is little published information.

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Methods and Material

Tracheal injection. By injecting the tracheal system with cobalt sulphi (Wigglesworth, 1950) it is possible to obtain permanent preparations in whic all those parts of the system which contain air are filled with the black depos while those parts which contain fluid remain colourless. For most purpos this method has proved entirely satisfactory; a tendency for the preparatio to fade has been got over by mounting in Gurr's neutral medium to whic 5 per cent. of the antioxidant propyl gallate (Progallin P of Nipa Laborator Ltd.) has been added. But the method has the disadvantage of giving granular deposit in the tubes (fig. 10, J (opposite p. 126)). A modification ha therefore been adopted for some purposes.

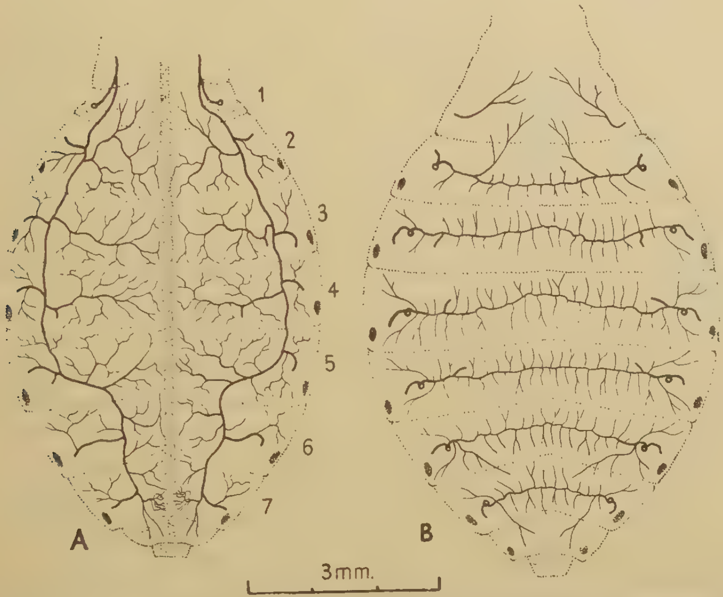
This depends on the fact that the cobalt naphthenate will react with 4-dinitroso-resorcinol (DNR) to give an intense orange-brown colour. Th tracheal system is filled with 33 per cent. cobalt naphthenate in white spir (light petroleum) as usual; the insect is then opened up and immersed i fixative freshly saturated with DNR. In order that the substances may intera the tracheal wall must be rendered permeable. This is effected by using fixative containing chloroform. (The effect of chloroform in increasing th permeability of the tracheae may perhaps afford evidence of the existence o a wax lining in the tracheal system.)

It is best to use a mixture containing a minimal amount of chloroform; too much is present penetration occurs rapidly in the larger tracheae and th finest tracheoles are unstained. The mixture generally used consists of ethano 80 c.c., 40 per cent. formaldehyde 20 c.c., picric acid 0·6 g. Immediatel before use 2 per cent. of chloroform is added and the mixture saturated wit DNR. The tissues are left in this for about 20 minutes, transferred to Bouin' aqueous mixture for 10 minutes, and then to a saturated solution of DN in water for half an hour.

The deep orange precipitate forms an apparently homogeneous layer (presumably a colloidal deposit) over the walls of tracheae and tracheoles (fig. 1 A, D, and E). If mineral acids are avoided it is quite permanent, and after th picric acid has been removed various counterstains can be used. One of th best counterstains consists in immersing in dilute iron alum (say 0·25 per cent. for 1–2 minutes, washing well in water, immersing for a few seconds only i dilute ammonium sulphide solution (see Wigglesworth, 1952), and the placing in a fresh saturated solution of DNR in water for 2 hours. This give a brilliant blue-green stain which provides a perfect contrast for the orang tracheae.

Insect material. Most of the observations and experiments have been mad on the sub-epidermal tracheae of the abdomen in *Rhodnius*. The tergites an sternites are separated by cutting along either side of the abdomen and ar then mounted flat. Since the tracheae below the epidermis run in on plane they lend themselves well to observations and measurements of a kinds.

Fig. 1 shows the arrangement of the abdominal tracheal system in the urth stage larva of *Rhodnius*. The first abdominal spiracles open dorsally low the wing pads. The remaining six pairs open on the sternites. In each gment a trachea arises close to the spiracle and runs across the ventral surce of the abdomen to anastomose with its fellow from the opposite side. he main trachea runs dorsally to join a lateral longitudinal trunk which tends throughout the abdomen. This gives off tracheae to the dorsal surface



IG. 1. Tracheal system in the abdomen of *Rhodnius* fourth stage larva. A, tergites with dorsal vessel in mid-line. B, sternites. Branches to the viscera have been cut short.

hich do not anastomose. As can be seen by comparing the two sides of g. 1, A there is much variation in the detailed arrangement of the branches. The visceral tracheae have been omitted from fig. 1; their points of origin an be seen as stout branches which terminate abruptly.)

These sub-epidermal tracheae lie for the most part between the lace-like t body and the epidermis. After injection and fixation it is possible to strip way the fat body, breaking through those few small branches by which it s supplied, so as to leave an unimpeded view of the epidermis and the tracheal ystem, which can then be stained and mounted whole.

SOME MEASUREMENTS OF THE TRACHEAL SYSTEM

Most of the observations and experiments have been made on the tracheae eneath the tergite of the fourth segment of the abdomen in the fourth stage arva. The main trachea divides repeatedly until it is reduced in diameter to bout 5 μ. Those branches which are going to supply the epidermis then pierce the basement membrane, so that all the smallest tracheae and the tracheol lie between the epidermal cells and the basement membrane (Wiggleswort 1933).

Fig. 2, A shows a typical tracheal ending. The terminal trachea usual measures 1·5–2 μ in diameter; in this example it measures 1 μ. It then brea up abruptly into two or three tracheoles which measure 0·7–0·8 μ in diamet at their commencement. Tracheoles of similar size are also given off from th sides of the larger tracheae throughout their course. (In this and subsequen figures only the formative cells of the tracheal system are shown: the epidermal cells beyond and the haemocytes on the surface of the basemen membrane are omitted.) The cell boundaries are not readily seen in th tracheal epithelium of the smaller branches but they show up clearly in silve preparations of the larger trunks (fig. 10, B).

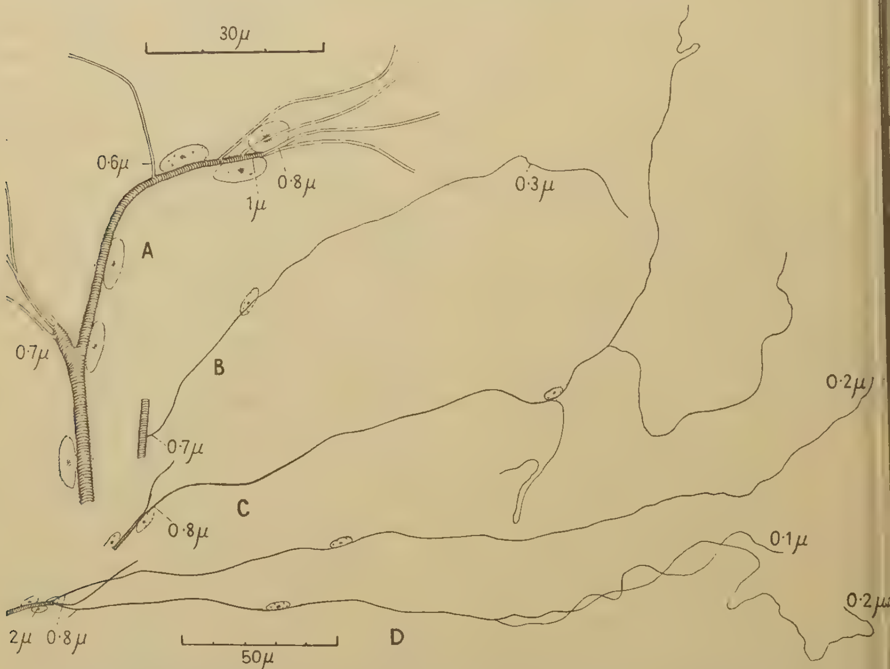


FIG. 2. A, tracheal ending below epidermis in fourth stage larva. B–D, single tracheoles wit their nuclei, drawn in full. The measurements show the diameter of the tubes at the point indicated.

At the termination of the trachea there are one or two nuclei loosely applie to the surface or lying between the points of origin of the tracheoles. As wi appear later, these nuclei belong to the trachea; they are not the formativ cells of the existing tracheoles. In fig. 2, B, C, and D several tracheoles hav been drawn in full to show their arrangement and dimensions; their measurements are summarized in Table 1.

TABLE 1. *Measurements of tracheoles in microns*

iameter at origin	*From origin to nucleus*	*Total length*	*Estimated diameter at ending*
0·8	70	345[1]	0·2–0·3
0·8	95	290	>0·2
0·7	55	195	0·2–0·3
0·8	75	280	0·2–0·3
1·0	70	280	0·2–0·3
0·8	70	305	0·2–0·3

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[1] At about half-way along, this tracheole gave off a fine branch, about 0·2 μ in diameter its origin, 100 μ in length, with a diameter at its ending apparently less than 0·1 μ.

The most frequent type is a single unbranched tube, about 250 μ in length, ith a single nucleus lying about one-third of the distance from the point of igin. The tube tapers gradually from a diameter of 0·7–0·8 μ to end in blunt rounded tip at a diameter of about 0·2 μ. Often the tracheole has side branch and sometimes more than one. In such cases the nucleus may lie the point of branching (fig. 2, C). Simple or branched tracheoles often arise om the sides of the tracheae (fig. 2, B); these likewise have a single nucleus. he tracheoles have these same dimensions in all the larval stages of *Rhodnius*.

The tracheoles lie proximal to the nuclei of the epidermal cells, close to e outer surface of the basement membrane; but just before they terminate ey often bend outwards to end between the epidermal cells, distal to the iclei. Occasionally a stained filament can be seen extending a little way eyond the rounded end of the tracheole.

The methods of injection employed, particularly the use of DNR and obalt naphthenate, reveal the form of the tracheal intima very clearly. They onfirm the view that the essential feature is the spiral folding of the lining embrane; the formation of a spiral thread is probably a secondary phenoenon resulting from the filling of the folds with cuticular substance (cf. . 135). In many of the smaller branches the folds are annular and not spiral ig. 10, A and E).

It has been shown with the electron microscope that spiral or annular folds xist equally in the tracheoles (Richards and Anderson, 1942; Richards and orda, 1950). After using the cobalt and DNR method of injection it is ossible to see these folds quite distinctly with the light microscope in many reparations. They are visible in tracheoles with a diameter of 0·6 μ and erhaps less.

NORMAL GROWTH AND MOULTING IN THE TRACHEAL SYSTEM

Maintained at 25° C., the fourth stage larva of *Rhodnius* moults 14–15 days fter feeding.

Fig. 3, A shows a tracheal ending at 7 days after feeding, at the height of itosis in the epidermis. The cells at the extreme end of the trachea are in rocess of multiplication to give rise to an elongated cluster of tracheal cells.

Fig. 3, B is at 8 days. The newly arisen tracheal cells are extending outwar to form a column. Many of them have filamentous outgrowths by means which they presumably draw themselves along. Some of these outgrowt may represent future tracheoles.

Fig. 3, C, also at 8 days, shows the column of tracheal cells with their nucl more widely separated, forming an elongated strand in which there is as y no sign of a lumen.

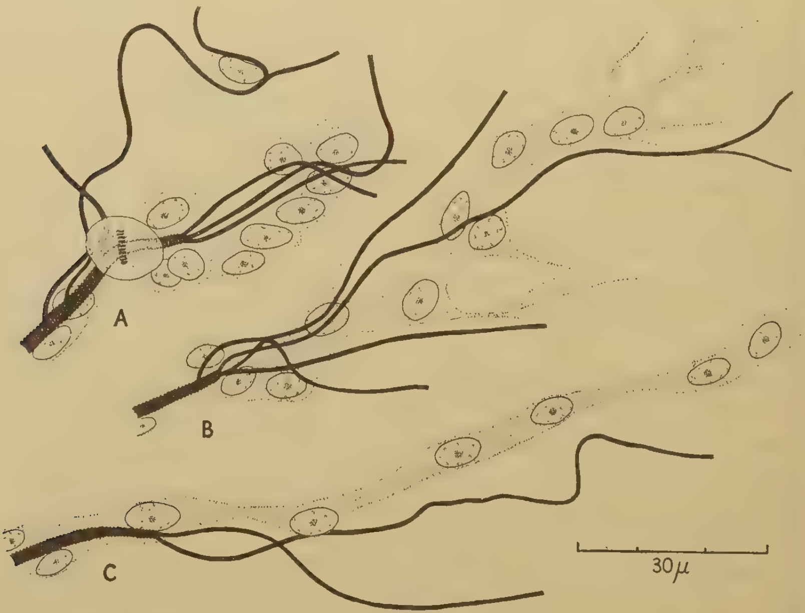


FIG. 3. A, trahceal ending at 7 days after feeding; tracheal cells multiplying to form a elongated cluster. B, at 8 days: the cluster of tracheal cells extending outwards. C, also a 8 days: tracheal cells fully extended to form a column beyond the tracheal ending. Existin tracheae and tracheoles filled with injection mixture.

Fig. 4, A and B show tracheal endings at 10 days after feeding. At this stag the new epicuticle of the abdomen has been laid down and is fully folded bu very thin. On the other hand, there is no visible separation of the cells from the tracheal walls even in the largest branches. Thus the formation of th epicuticle over the surface of the body precedes its formation in the trachea system. But in the columns of tracheal cells forming the new tracheae the lumen is now fully developed, though as yet there is no spiral folding of the wall

The new tracheae, which measure about 1·5–2 μ in diameter, show almost no tapering throughout their entire course. The tracheoles are still in process of formation. At their endings the formative cells show numerous branche filaments (fig. 4, B). These are not visible when development is complete they are presumably in the nature of pseudopodia and concerned with outward migration of the growing tracheole. Only a few of these filaments become canalized (cf. Keister, 1948).

(The basement membrane is sometimes said to be produced by the tracheal lls, but it is quite clear that in *Rhodnius* they do not contribute to it. The ses of the epidermal cells give off anastomosing filaments which are attached or fuse with the basement membrane (Wigglesworth, 1953). The ramifica-ons from the tracheal cells described above lie distal to these filaments and e thus separated from the basement membrane.)

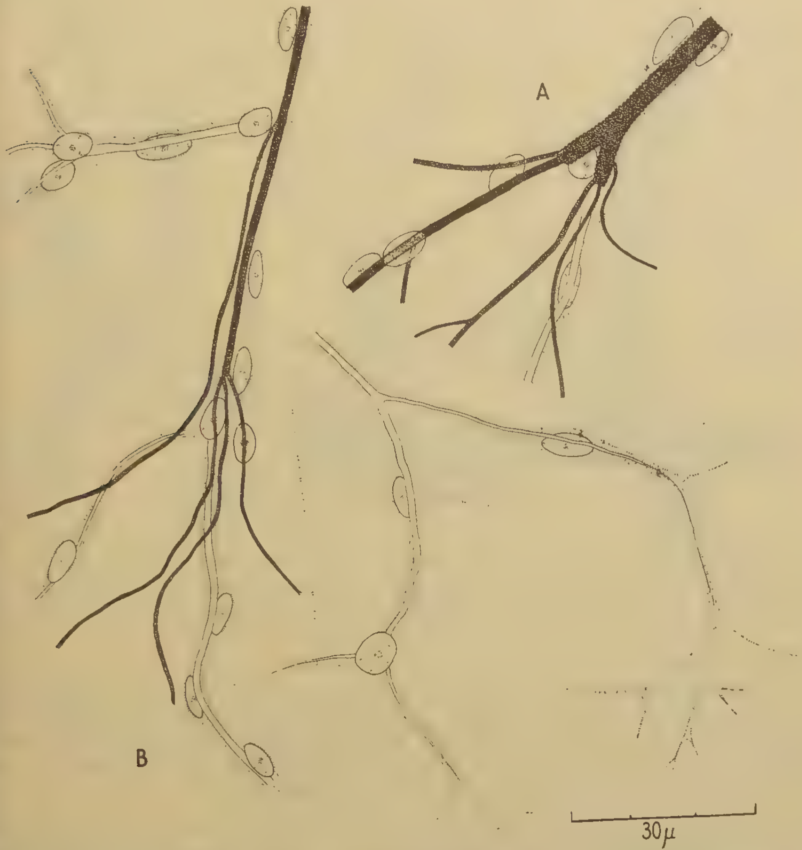


FIG. 4. A, tracheal ending at 10 days after feeding: existing tracheae and tracheoles injected; new trachea has colourless lumen. B, also at 10 days: lumen of new tracheae formed and lumen of new tracheoles forming.

Fig. 5 shows a tracheal ending at 12 days. By this time the new tracheal intima has been laid down. In the larger branches there is a wide space between the old and the new walls and the spiral folds have already appeared in the new intima; but in the smaller branches represented in fig. 5 the new walls are still smooth and are only slightly separated from the old tracheal cuticle.

In the existing tracheoles there is no detachment of the lining. Consequently, at the point of origin of the tracheole, where the new tracheal wall has separated from the old, the delicate lining of the tracheole crosses the gap between the two membranes (fig. 5, *a*). Here it lies in the moulting fluid, freely expos and devoid of cytoplasmic covering. This short length of tracheole, usua not exceeding 1 μ, is the only portion that is shed at moulting. Otherwise t tracheoles persist unchanged from one instar to the next.

Fig. 6 shows tracheae a few minutes before moulting. These were dra from preparations made when the insects had taken up their inverted attitu

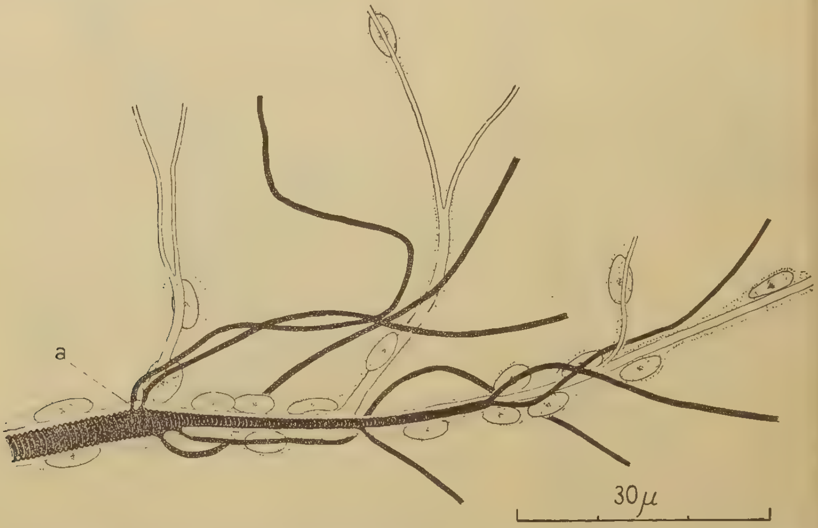


Fig. 5. Tracheal ending at 12 days after feeding: new cuticle of existing tracheae separate from the old cuticle by space filled with moulting fluid; lumen of new tracheae and tracheole now continuous with this space. *a*, short length of existing tracheoles, with walls partiall collapsed, crossing the gap between the old cuticle and the new.

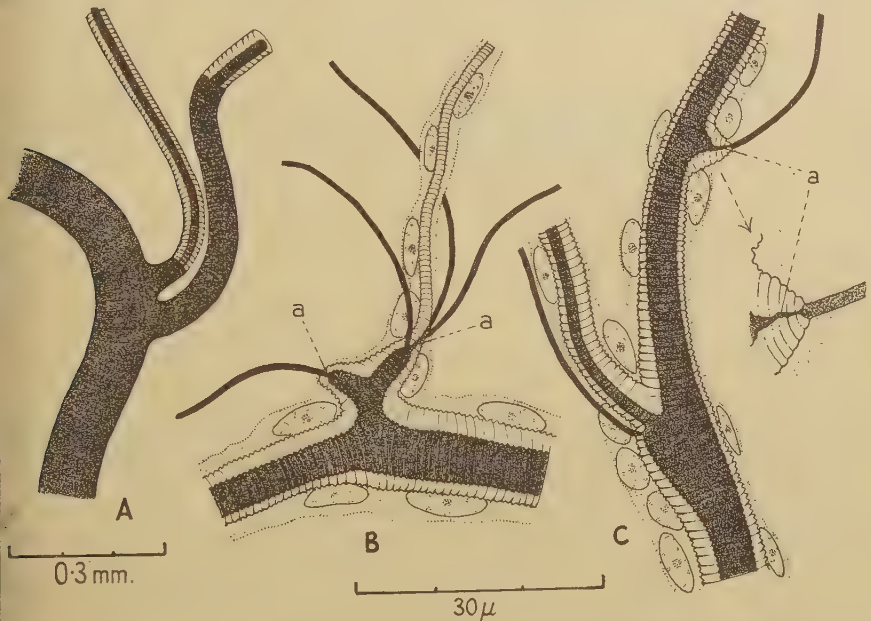
preparatory to moulting; the moulting fluid was being absorbed, and air wa present below the cuticle. As seen in fig. 6, A, air was already replacing th moulting fluid in some of the larger new tracheae, but all the smaller branche still contained fluid in the space outside the old trachea.

The new tracheal walls are fully formed and the spiral folding complete The old existing tracheoles are filled with the injection fluid, showing that th connexion with the old tracheal lining is still unbroken; but these connexion are becoming excessively tenuous and in mounted preparations they hav usually collapsed (fig. 6, B, *a*). The newly formed tracheae and tracheoles stil contain fluid.

As soon as the cuticle of the thorax splits and the insect begins to leave th old skin, the linings of the old tracheoles rupture at the point where they joi the new tracheal wall, the old linings are withdrawn, and the entire syste fills with air as the tracheal fluid is absorbed.

General conclusions. The tracheal system in *Rhodnius* does not grow by th conversion of tracheoles into tracheae. The tracheoles once laid down persis from one instar to the next; presumably they remain functional throughou life of the insect and at no stage do they shed their lining membrane. This ne process was observed by Keister (1948) in the last larval moult and in pupal moult of *Sciara*; whereas in the earlier larval stages of this insect entire lining of the tracheal system is shed.

The new tracheae in *Rhodnius* arise chiefly from growing points at the ends the existing tracheae and to a lesser extent by lateral outgrowths along the



IG. 6. Tracheae at 15 days, a few minutes before moulting. A, large tracheae close to the iracles: in the largest branches the injection mixture fills the new tracheae, showing that ese now contain air; in the smaller branches moulting fluid is still present and only the old acheae are injected. B and C, small tracheae and tracheal endings: new tracheae and space etween old and new cuticles still filled with moulting fluid. *a* shows partially collapsed tracheoles crossing the gap between the two cuticles.

ourse of the tracheae. Thus the tracheoles which at one stage form the erminal ramifications of a tracheal stem get left behind, like the side branches rising from a node in a plant. It is sometimes possible to recognize these odal points along the course of a trachea: they show as step-like reductions the diameter of the trachea with a group of tracheoles arising from each figs. 7 and 10, D).

Fig. 8, which represents the dorsal trachea of the fourth abdominal segment n a first stage larva and a second stage larva (necessarily different individuals), ikewise illustrates the enlargement of the tracheal system by the outgrowth f new tracheae and tracheoles, while the existing tracheoles remain intact.

When the new trachea is long it is formed by a column of cells (as in ig. 3 or fig. 4, B); but where it runs only a short distance of 15–30 μ before it

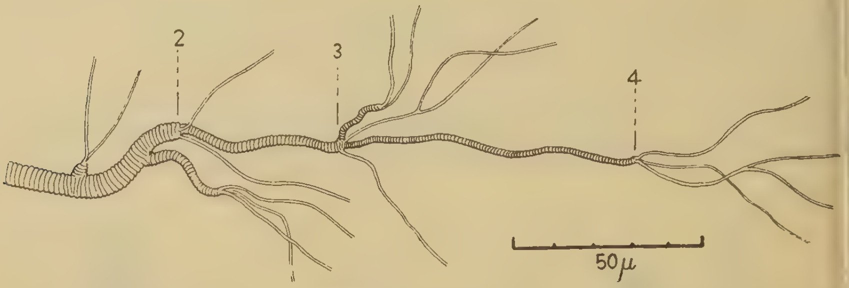


FIG. 7. Tracheal ending in fourth stage larva showing 'nodal' points with tracheoles arisi from each. The numbers indicate the points of termination of the trachea in the second (third (3), and fourth instar (4).

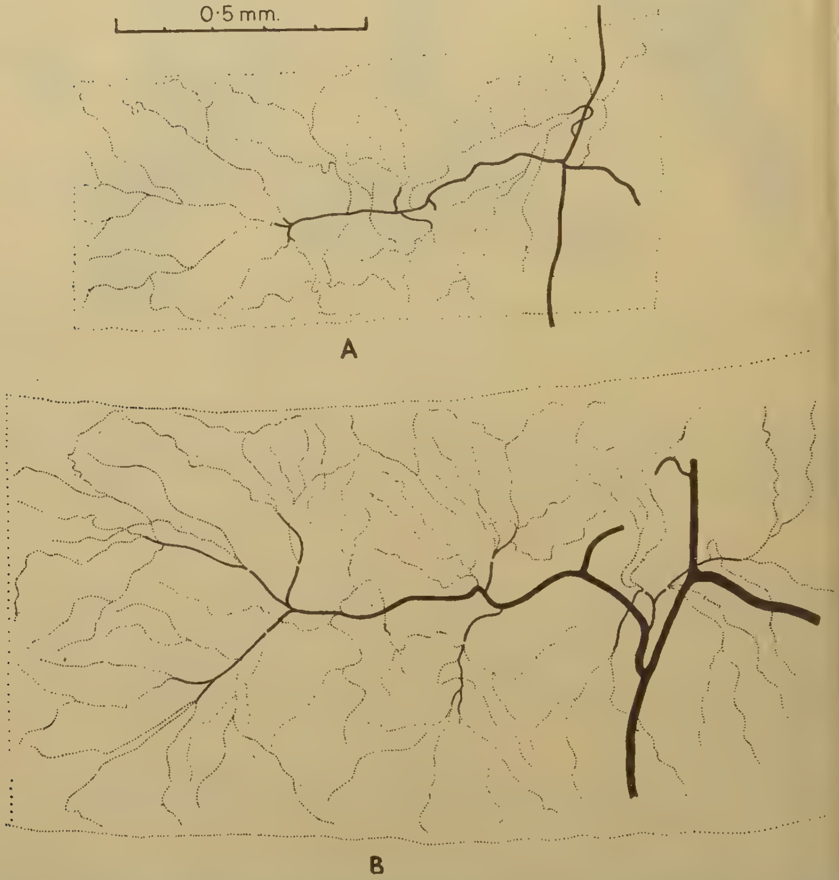
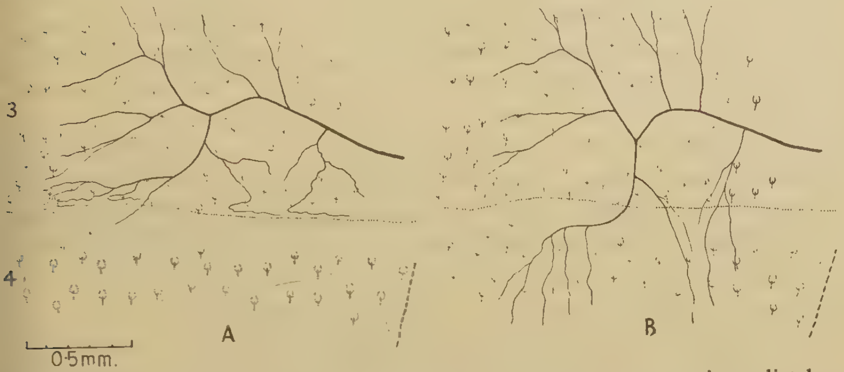


FIG. 8. A, tracheal supply to fourth tergite in first instar larva: tracheae continuous, tracheol dotted. B, the same in second instar larva: the breaks in the continuous line mark the poin at which the new tracheae, developed in the second instar, have arisen.

aks up into tracheoles, as seen in the lateral branches arising in fig. 5, the ole length may be formed by a single cell—as is always the case in each cheole. The new trachea always shows a uniform diameter of 1·5–2 μ. \s Keister (1948) points out, there is no difference in principle between the des of development of tracheae and tracheoles. But, at least in *Rhodnius*, re seems to be an absolute distinction between the two: the tracheae shed ir linings at moulting, the tracheoles do not. If it is justifiable to regard this



G. 9. Migration of tracheoles observed in living fourth stage larva. A, immediately after ction of the main trachea to the fourth tergite. B, 14 days later. The dotted line marks the tersegmental boundary; the broken line shows the incision through which the trachea was cut.

a constant distinction between tracheae and tracheoles, then one might say at in *Sciara* as described by Keister (1948) true tracheoles are not formed ntil the last larval stage.

MECHANISM OF TRACHEATION WITHOUT MOULTING

Various methods have been used for depriving a region of the epidermis of s tracheal supply and observing its restoration. A simple method, causing minimum of injury to the tissues, consists in making a minute incision at the de of the fourth tergite, and by means of a needle with a hooked point, aring through the trachea to that segment near its origin from the lateral unk. The whole of the integument of the fourth tergite as far as the mid-line thus deprived of its tracheal supply.

If the upper surface of the abdomen is now varnished with shellac, it is ossible, in a strong incident light, to obtain a good view of the tracheae and rger tracheoles. They can be drawn with the camera lucida and their position cated by reference to the plaques and bristles in the overlying cuticle.

The results are striking: the tracheoles migrate inwards from the segments ehind and in front, and from the opposite side of the fourth segment. In ome experiments they may migrate as much as one millimetre, drawing the racheae after them.

Fig. 9 shows the result of one such experiment. The trachea of the fourth segment was cut 24 hours after feeding. Fig. 9, A shows part of the trachea the third segment immediately after the operation. At 3 days after secti there was a very slight displacement backwards of the tracheae of the th segment. At 7 days the main branch of this trachea had been drawn right ba on to the fourth segment and the tracheoles could be seen running backwar across the fourth tergite. At 14 days (fig. 9, B) this movement had extend a little farther. In this final position the endings of the tracheoles (as prov subsequently by injection) had moved half-way across the fourth segment supply the anterior half of this tergite (about 700 μ from their starting-poin and the tracheoles from the fifth segment had similarly migrated forwards supply the posterior half (see also fig. 10, F and G).

We have seen that the smaller tracheae and all the tracheoles run on t distal side of the basement membrane, between the bases of the epiderm cells. In the course of this extensive displacement, during which the tracheol may be drawn into nearly straight lines, it is obvious that they must mo laterally across the bases of the epidermal cells. This would indicate that t epidermal cells are readily detached from the basement membrane—as inde is evident (*a*) during normal mitosis in the epidermis, and (*b*) during t migration of epidermal cells towards a point of injury (Wigglesworth, 193

After incision of the integument the epidermal cells migrate from the su rounding region to the margin of the wound (Wigglesworth, 1937). But th response does not influence cells more than about 200 μ away. It is quite cle that the tracheoles in the experiments here described are actively migratin to the region of deficient oxygenation: they are not being carried passive along by movements of the epidermal cells. Indeed, as can be seen in fig. migration of the tracheoles takes place into the uninjured region deprived its tracheal supply and not towards the wound.

In other experiments, after section of the main trachea to the fourth tergit the corpus allatum plus the corpus cardiacum removed from a fifth insta larva were implanted through a small incision in the middle of the zone tha had been deprived of its tracheal supply. This leads to a great increase in th

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FIG. 10. A, small tracheae showing mainly annular folding of cuticle. Cobalt naphthenate an dinitroso-resorcinol (DNR) method. B, tracheae showing nuclei and cell boundaries. Silve staining. C, small tracheae and tracheoles in thoracic gland. Cobalt and DNR. D, trache showing 'nodal points' (marked by arrows) where there is an abrupt reduction in size and th terminal tracheoles of an earlier instar can be seen arising. Cobalt and DNR. E, trachea showing folding of the cuticle, mainly annular in the small branches. Cobalt and DNR. tracheae on one side of the third tergite of a fourth stage larva. The vertical dark shade is th dorsal vessel. (The tracheae of the opposite side are intact but the injection has failed. Cobalt sulphide. G, tracheae on one side of the fourth tergite in the same insect as F. Th trachea of the opposite side had been cut 10 days earlier and the tracheoles have migrate across the mid-line. Cobalt sulphide. H, part of fourth tergite of fifth stage larva in whic a corpus allatum had been implanted in the fourth instar (after cutting the tracheal supply The vertical dark shade to the left is the dorsal vessel. Note the enlargement of the newl formed tracheae as they approach the implant to the right. Cobalt sulphide. J, newly forme tracheoles in the neighbourhood of an implanted corpus allatum, showing the highly con voluted course they pursue. Cobalt sulphide.

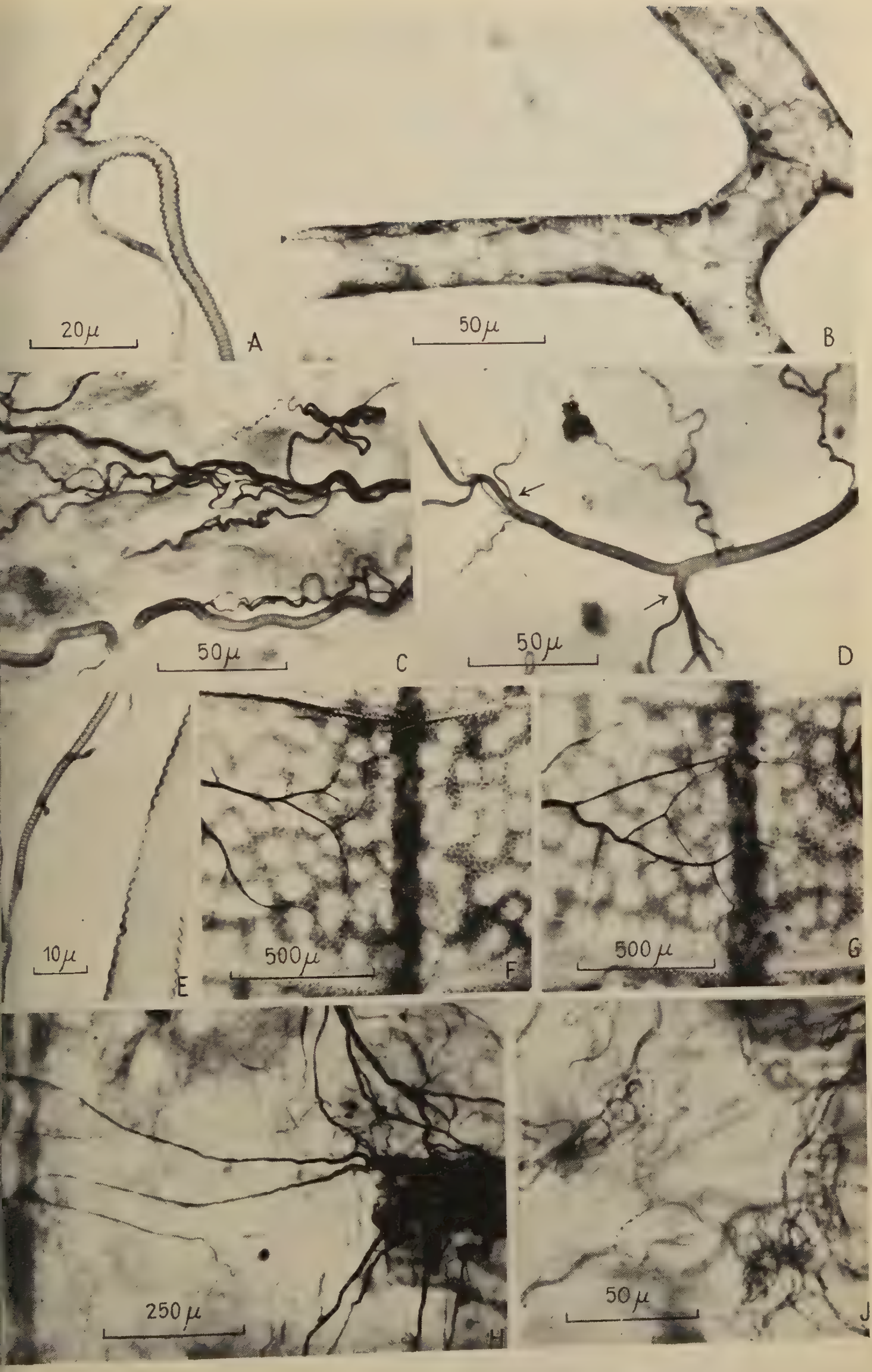
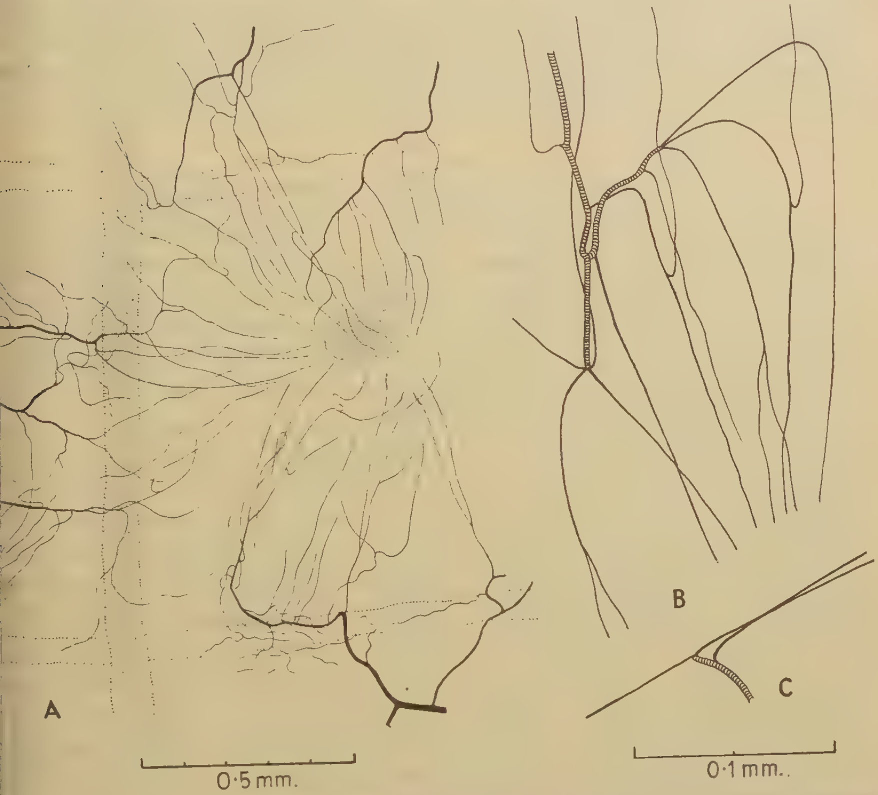


Fig. 10

V. B. WIGGLESWORTH

gen requirements and a very active migration of tracheoles, which now verge upon the implant. Fig. 11, A shows the result when the tracheal tem was injected at 12 days after the operation. The line of the aorta and the intersegmental boundaries can be taken as the approximate starting-int of the tracheoles at the outset. They have moved about 400–700 μ.



IG. 11. A, migration of tracheoles towards implanted corpus cardiacum and corpus allatum, fter section of the main trachea to this segment. Tracheae injected 12 days after the implanta-on. Dorsal vessel shaded; intersegmental boundaries dotted. B, tracheoles converging upon nplanted corpus allatum, showing tension exerted by migrating tracheoles. C, detail of tracheoles pulling in opposite directions.

heir endings run over the surface of the corpus allatum but they have not een observed to penetrate between the cells of this organ.

(It must be emphasized that although the position of the tracheoles in fig. 1, A has been represented fairly accurately with the aid of the camera lucida, ll but the largest branches are very much finer than here depicted. This nakes the tracheal supply appear much richer in this figure than it really is.)

Tracheae and tracheoles running in the opposite direction do not take part n this movement, so that tension is exerted upon them by the migrating racheoles. Examples of this can be seen at various points in fig. 11, A, and at larger magnification (from another preparation) in fig. 11, B. Sometimes

the tension is so great that the tracheoles are drawn taut into a straight lin
as seen in fig. 11, c.

At the most active period of movement, 5 or 6 days after the operation, t
tracheal system has been injected and the cells deeply stained in an effort
observe the changes in the tips of the tracheoles. Presumably there must
pseudopodial processes extruded from the ends of the formative cells of t
tracheoles, like those visible during their initial growth (fig. 4, B); but it h
not been possible to observe these with certainty. The position of the trache
nucleus, about one-third of the distance along the tracheole (fig. 2, B–
remains unchanged.

MECHANISM OF TRACHEATION DURING MOULTING

If the *Rhodnius* larva is decapitated 1 day after feeding, its growth is arreste
and it does not moult (Wigglesworth, 1934). If the operations described
the preceding section are carried out on such an insect it remains alive f
several months and extreme degrees of tracheal migration may occur. But i
the absence of growth and moulting no tracheation takes place apart fro
that brought about by the migration of existing tracheoles. Indeed, in view
the fact that the entire tracheal system is lined by an unbroken cuticular membrane, it is difficult to see how new air-filled tracheal tubes could be forme
in the absence of a moult.

But, in the normal insect, added to this process of migration there is a
extensive formation of new tracheae and tracheoles which become functiona
when they are filled with air at the ensuing moult. This process is simply a
exaggeration of the tracheal growth which takes place at normal moulting, bu
the new tracheae and tracheoles are much longer and more numerous than usua

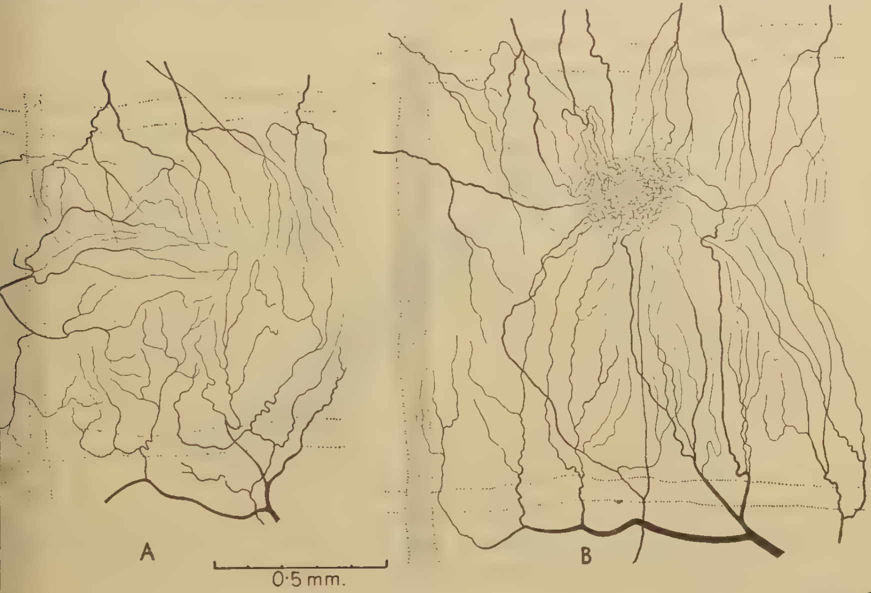
The epidermis and tracheae over a wide area on one side of the fourt
tergite of a third stage larva were destroyed by burning with a heated glas
rod. When this larva had moulted to the fourth stage the tracheal supply t
the injured fourth tergite was found to have been made good from the neighbouring segments.

We have seen that the new tracheae always measure 1·5–2 μ in diamete
and arise from a nodal point where there is an abrupt contraction in size an
where a group of tracheoles, the terminal tracheoles of the preceding instar
are given off. It was thus possible to make measurements of the new growt
shown by approximately corresponding tracheal branches on the normal an
the injured side. The results are given in Table 2, all the measurements bein
in microns.

TABLE 2

	Normal side	*Injured side*
Length of trachea:	80; 105	225; 340; 730
Length of tracheoles:	200; 210; 240; 255; 255; 280	420; 450; 420; 540
Diameter of tracheoles at origin:	0·7–0·8	0·9–1·0

The very long new trachea of 730 μ ran this entire distance without givin any tracheoles or tracheal branches; and it showed another feature which haracteristic of regenerated tracheae when they exceed a certain length—it ited as it approached its termination. In this instance the trachea started h a normal diameter of 2 μ. It ran 350 μ enlarging very slightly to 2·5 μ. then enlarged more rapidly and on reaching a length of 475 μ it had uired a diameter of 6 μ. After a further 100 μ it had contracted down to iameter of 3 μ, where it gave rise to two terminal branches of 2 μ diameter ich ran a further 50 μ before breaking up into tracheoles.



G. 12. A, tracheae and tracheoles invading fourth tergite of a fifth stage larva after section of main trachea to this tergite in the preceding instar. B, the same, in a larva in which a pus allatum had been implanted. Only the tracheae and some of the larger tracheoles have been drawn.

Fig. 10, H shows a number of new tracheae dilating in this way as they proach their objective. In this preparation some of the new tracheae were least one millimetre in length.

The abundance of newly formed tracheae is dependent on the oxygen quirements of the region to be supplied. Fig. 12, A shows the new tracheae nd a few of the larger tracheoles) supplying the fourth tergite after section the trachea to this segment in the preceding instar. Fig. 12, B shows the new cheal supply after a similar tracheal section combined with implantation a corpus allatum. Not only are there many more new tracheae converging on the implant, but as they approach the corpus allatum they branch peatedly and pursue a highly convoluted course. Some of the tracheoles netrate deeply into the organ, insinuating themselves between the cells st as in the normal gland. Fig. 10, H shows another example; the richly cheated mass to the right is an implanted corpus allatum. Fig. 10, J shows a group of highly convoluted tracheoles in the neighbourhood of such implant.

OXYGEN DEFICIENCY AS A STIMULUS TO TRACHEAL MIGRATION

The most obvious conclusion from the observations so far described is th lack of oxygen provides the stimulus to the migration of existing tracheo and to the growth and extension of new tracheae and tracheoles.

The effect of a low oxygen tension on the movement of existing tracheo was demonstrated as follows. A small incision about half a millimetre lo

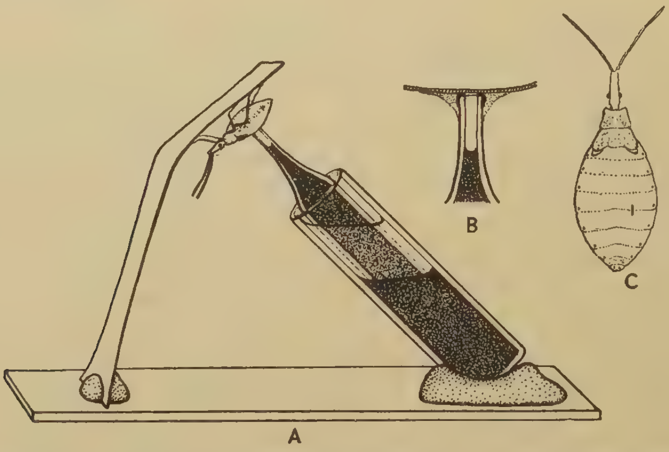


FIG. 13. A, method of exposing the back of a *Rhodnius* larva to zero oxygen tension; descri tion in text. B, tip of the tube containing pyrogallol, sealed with cigarette paper, secured cuticle with paraffin wax. C, position in which the cuticle is cut.

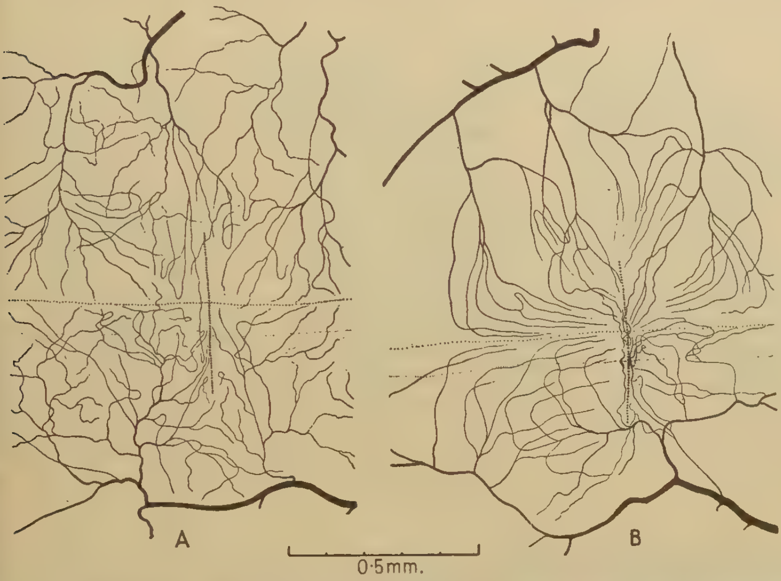
was made in the integument of the intersegmental region between the four and fifth tergites of a series of fourth stage larvae at 1 day after feedin (fig. 13, C). Half were left as controls exposed to the air. In the remainder th region of the incision was exposed to zero oxygen tension.

A tapering glass tube was ground level at the tip and this was sealed wi a diaphragm of cigarette paper secured with shellac. The diaphragm was the brought into contact with the incision and held in position with paraffin wa (fig. 13, B). The tube was now filled with alkaline pyrogallol (10 per cen pyrogallol in 10 per cent. NaOH), a small space being left between the liqu and the paper diaphragm. The inverted tube was placed in a phial of pyr gallol covered with a thick layer of liquid paraffin (fig. 13, A).

At 1 week after the operation the insects were injected and the reaction the tracheoles around the incision compared in the two lots. Fig. 14 sho typical results. In the insect exposed to the air (fig. 14, A) only those tracheol in the immediate vicinity of the incision appeared to be influenced by it; the was in fact only a very small amount of migration of tracheoles towards t wound. When the incision was exposed to zero oxygen tension (fig. 14, B) tracheoles in the surrounding region migrated towards the wound. In some ces, tracheoles as much as 400–500 μ away were affected.

OXYGEN DEFICIENCY AS A STIMULUS TO TRACHEAL GROWTH

f the new growth of tracheae is induced by the low oxygen tension in the ion of their endings it should be possible to observe an increase in the mber of tracheal branches in insects reared at low partial pressures of ygen. *Rhodnius* larvae have been reared in flasks, immersed in a water bath 26° C., with gas mixtures of known composition passed through them. At per cent. oxygen many of the first stage larvae died during moulting; but ey have been successfully reared to the fifth instar in 7·5 per cent. and in per cent. oxygen in nitrogen.



IG. 14. A, tracheae and tracheoles in the region of an incision of the integument (shown as vertical dotted line), exposed to air; 7 days after operation. B, the same, with incision exposed to zero oxygen tension.

The tracheal supply to the abdomen is too variable to provide really con-incing evidence that a reduction in oxygen tension causes a change in the rm or number of tracheae. Comparative observations were therefore made n organs which normally have a fairly constant arrangement of tracheae.

The fused ganglia of the meso- and meta-thorax and abdomen are normally upplied by four tracheae, an anterior pair coming from the mesothoracic piracles, and a posterior pair coming from the metathoracic spiracles. Fig. 15, A epresents this tracheal supply as it is seen in the fifth stage larva. As the main racheae approach the ganglia they divide into dorsal and ventral branches. The dorsal branches vary in the amount of the fused ganglia which they supply and in the detailed arrangement of the terminations, but the gene arrangement is fairly constant. No branches from the abdominal trache supply the ganglia; the large nerves which run backwards are accompani by slender branches derived from the thoracic tracheae.

In larvae reared at reduced partial pressures of oxygen the tracheal supp to the thoracic ganglia is much less regular in its arrangement, and the numb of large branches is increased. The large nerves to the abdomen commo acquire a tracheal supply from the transverse trachea of the second abdomi

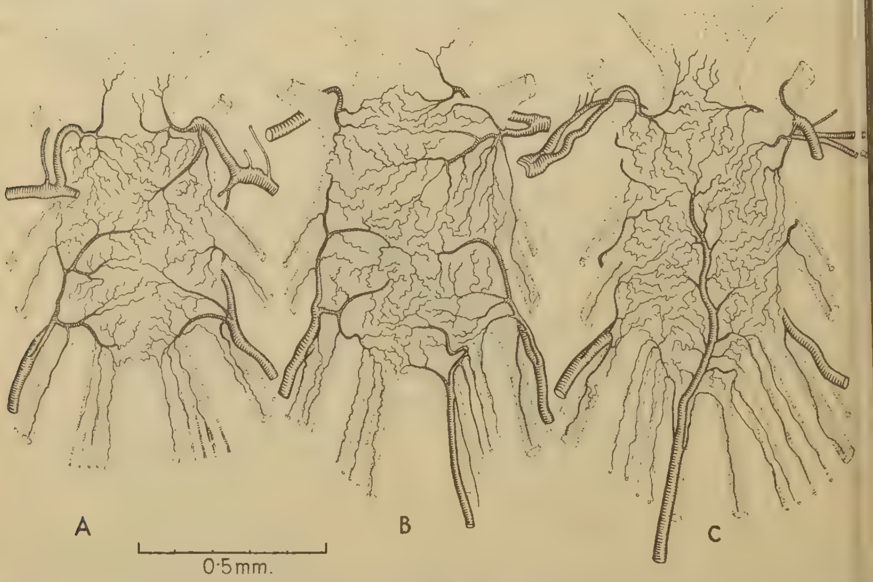


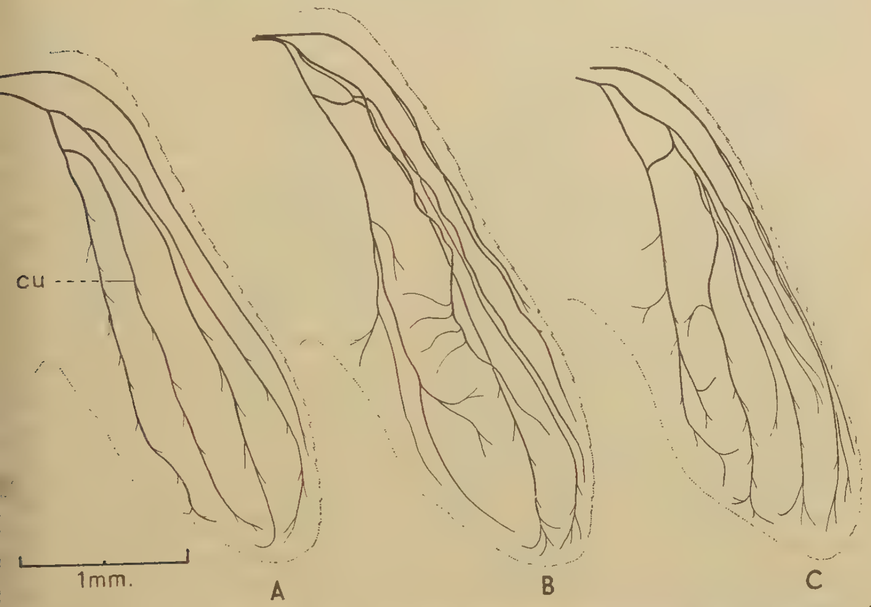
Fig. 15. A, fused thoracic and abdominal ganglia of fifth stage larva reared in air. B, the sam from larva reared in 10 per cent. oxygen. C, the same from larva reared in 7·5 per cent. oxyge

segment and these tracheae commonly run forwards along the nerves t provide an additional supply of large branches to the thoracic gangli Fig. 15, B shows a typical example in a fifth stage larva reared from the eg in 10 per cent. oxygen. Fig. 15, C is from 7·5 per cent. oxygen; it shows th development of a very large trachea derived from the second abdomin segment providing the tracheal supply for a large part of the fused ganglia.

TRACHEATION AND WING VENATION

Similar effects are produced on the tracheal supply to the wing lobes. I insects reared in air the tracheation of the wing is very constant (fig. 16, and consists of five main tracheae running a longitudinal course. In insect reared in 7·5 per cent. oxygen the arrangement of the tracheae is les regular; longitudinal tracheae are more robust and their number may b increased to eight or nine, with a further increase in the smaller branche (fig. 16, B and C).

It was of interest to observe the arrangement of the wing veins in the adult
gs derived from these larvae. In hemimetabolous insects the tracheae to
wing are commonly developed before the veins are formed and they are
erefore often regarded as determining the course of the veins (Snodgrass).
t, as can be seen in fig. 17, B, C, and D, despite the anomalous trachea-
n of the wing lobes in the fifth stage larva, the general arrangement of the
ins in the resulting adult is more or less normal.



IG. 16. A, tracheae to wing lobe of normal fifth stage larva reared in air. B and C, the same in larvae reared in 7·5 per cent. oxygen. *cu*, trachea of cubitus.

That would suggest, as has been claimed by Henke (1953) and others, that
ne primary component in the venational pattern of the adult wing is the
ystem of lacunae; in normal development the tracheae merely provide a con-
enient indicator of where the lacunae run. It is obvious in fig. 16, B and C that
ven where the number of longitudinal tracheae has been increased by expo-
ure to low oxygen tensions, they come to lie for the most part along the usual
hannels. This is even more evident after the insect has moulted to the adult
tage (fig. 17, B–D).

And it is obvious from fig. 17 that a given vein may receive its tracheal
upply from very different sources (cf. the wing of *Ephestia* illustrated by
Henke and Berhorn, 1946). This is most clearly shown in the case of the
ubitus (fig. 17, *cu*). It frequently happens (though not invariably) that in
ifth stage larvae reared in 7·5 per cent. oxygen the fourth longitudinal trachea
(the precursor of the cubitus) is absent. That is so in fig. 16, B and C. In the
normal adult wing (fig. 17, A) this trachea can be seen running the whole length

of the cubitus. In fig. 17, B it is present, though reduced in length, and t cubitus has developed normally. But in fig. 17, C and D this trachea is absen the cubitus has received its tracheal supply in the form of small branch from neighbouring tracheae. In the proximal half of the wing the vein h developed normally; but in the distal part it is incomplete: those segmen which have not received an adequate tracheal supply have failed to develo

Conversely, it can be seen in fig. 17, D that in the distal half of the win where relatively large tracheae run across the wing to reach the position of t

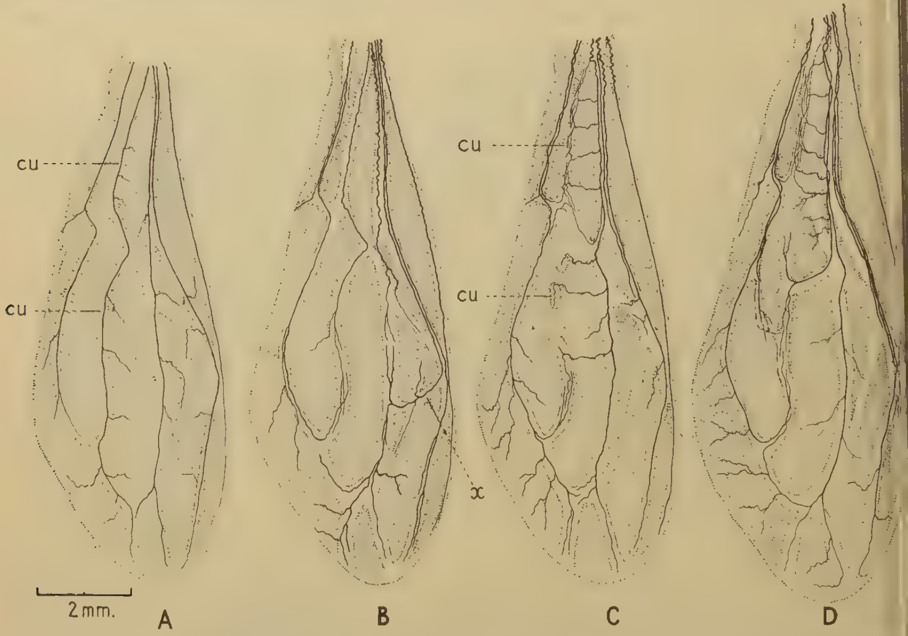


FIG. 17. A, veins and tracheae in wing of normal adult reared in air. B–D, the same in adul reared in 7·5 per cent. oxygen. *cu*, cubitus.

cubitus, rudimentary veins (or at least unpigmented zones resembling vein may be laid down around them. The same is seen towards the costal regio of the wing in fig. 17, B, *x*.

These observations would suggest that not only is an adequate trache supply necessary for the development of veins, but tracheae running a abnormal course may sometimes evoke at least rudimentary vein formatio around them. (This material and these methods will be used in a detaile experimental study of tracheation and venation in the insect wing at prese being undertaken in this laboratory by Mrs. J. Wells.)

DISCUSSION

(i) *Growth and moulting of the tracheal system*

Throughout all the larval stages in *Rhodnius* the tracheal system increase its extent at moulting by the outgrowth of new tracheae from the terminatio the existing tracheae, the cells required for this purpose arising by the ltiplication of the epithelial cells around the tracheal ending. Each tracheole, ich is usually 200–300 μ long and which may have one or two branches, is l down within a single cell the nucleus of which comes to lie about one-third the way along its length.

Once the tracheole is laid down it seems to persist unchanged throughout : life of the insect: its lining membrane is not shed at moulting and it does t become converted into a trachea. This persistence of tracheoles was served by Keister (1948) during pupation and adult formation in *Sciara*. *Rhodnius* it occurs throughout life, so that in this insect there is an absolute stinction between 'tracheae' which shed their linings and 'tracheoles' which not. This conclusion is opposed to that of Richards and Korda (1950), sed on the structure of the tracheal cuticle, that there is no real distinction tween tracheae and tracheoles (see p. 125).

The literature on the mode of formation of tracheoles has already been fully viewed by Keister (1948); the observations recorded in the present paper ree exactly with those made by this author in *Sciara*.

When the new tracheal membrane is first laid down at moulting it is smooth d is little larger than the cuticular membrane of the preceding instar. The w trachea then increases in diameter and at the same time the membrane thrown into folds which gradually deepen. There can be little doubt that ese spiral and annular folds result from an expansion in area of the newly rmed cuticle—such as is seen in the epicuticle of the body surface (Wigglesorth, 1933). The formation of the so-called 'taenidium' or spiral thread is oubtless a secondary phenomenon resulting from the deposition of cuticular aterial within these folds. It is of interest to note that when the cuticular ibstance of the old tracheae has been digested and the lining membranes are eing withdrawn, the folds are to a large extent smoothed out and the 'spiral iread' has disappeared.

i) *Migration of tracheoles to an area of deficient oxygenation*

The tracheoles are by no means inert structures. They are capable of active igration into regions of deficient oxygenation and are thus able, to some xtent, to make good deficiencies in tracheal supply in the absence of growth nd moulting in the tracheal system.

The reactive structure is presumably the tip of the tracheole, often situated o0 μ or more from the tracheole nucleus. The movement is presumably moeboid in character, resembling that of epidermal cells migrating towards site of injury (Wigglesworth, 1937). The resulting distortion of the tracheae hows that the tension exerted by the migrating tracheoles can be considerable.

For the moment it has been assumed that lack of oxygen provides the timulus of attraction for the tracheoles. But it is possible, of course, that some econdary effect of a reduced oxygen supply, such as the formation of acid netabolites, may provide the immediate stimulus. This will require further nvestigation.

It may be noted that when the trachea supplying one of the tergites *Rhodnius* is cut there are certain visible changes in the epidermal cells. Th are often vacuolated and pale-staining in comparison with the normal region and they contain much less red pigment and fewer white granules (s Wigglesworth, 1933).

(iii) *Increased growth of tracheae in response to oxygen lack*

The great increase in the formation and outgrowth of new tracheae whic takes place at moulting (after the interruption of the tracheal supply or th implantation of organs with high oxygen requirements) has likewise bee attributed in this paper to the direct effect of oxygen lack; but it may equall well result from some secondary change.

Whatever the immediate stimulus, it causes enormously increased mitos at the tracheal endings leading to the formation of more and longer trache and tracheoles. In addition, the outgrowing tracheoles pursue an excessive convoluted course in the neighbourhood of the oxygen deficient region. Th reaction might repay closer study: it invites comparison with the 'klinokinesi of an organism responding to a diffuse stimulus (Fraenkel and Gunn, 1940 Wigglesworth, 1941).

The same considerations apply to the increased formation of new trache in insects reared in low concentrations of oxygen. Insects keep their spiracle closed most of the time and open them only sufficiently to meet the oxygen requirements. They can therefore compensate for reduction in th oxygen concentration of the air by opening the spiracles more frequentl (Wigglesworth, 1935). It may be for this reason that there is no obvious chang in the tracheal supply to the abdomen in *Rhodnius* larvae reared in 7·5 per cen oxygen.

It may be only in the head and thorax that the much higher oxygen requirements cannot be made good in this way, so that some increase occurs in th supply of large tracheal branches. It is worth noting that under conditions o low oxygen tension the thoracic ganglia draw their extra supply from th abdomen. No such increase in tracheal development takes place in *Rhodniu* larvae reared in air containing 10 per cent. carbon dioxide. This gas mixtur must result in a greatly increased acidity in the tissues; it will also ensure tha the spiracles are held permanently open.

The net result of an increase in the number of large tracheal branches wil be to increase the average cross-section of the tracheal system and so to increas the rate of diffusion of oxygen from the spiracles and thus to raise the partia pressure of oxygen at the tracheal endings (Krogh, 1920).

Little information exists in the literature on the adaptive development o tracheae in response to physiological stimuli. But there are examples o exaggerated branching in the tracheal system of host insects to provide fo the respiratory needs of the eggs or larvae of parasites; and such activation o the tracheal epithelium has been attributed to the metabolic demands of th parasite (Thorpe, 1936; Simmonds, 1947).

) *Wing venation and tracheation; patterns of tracheal branching*

The present observations on the tracheation of the thoracic ganglia and the ngs raise the general question of what determines the constant pattern of cheal branching. Is there a predetermined pattern of tracheal growth, or is s pattern merely induced by the reactions of the growing tracheae to the mands of the other organs?

No final answer can yet be given to this question. Descriptions of the tracheal stem in various species of Ephemeroptera, for example, show a remarkable nstancy in anatomical arrangement (Landa, 1948). In *Rhodnius* the anatomy the tracheal branches in the abdomen is highly variable. In the thorax and ngs, where the anatomy of the organs is fixed, the tracheal pattern is far ore constant. But it is subject to large changes when the oxygen supply is duced.

So far as they go these observations suggest that the tracheal pattern is condary to the anatomical pattern of the other organs. Such a conclusion ould be consistent with the whole physiology of tracheal growth as described this paper. On the other hand, after a detailed study of the tracheation of e insect wing, Henke (1953) does not believe that the pattern of tracheal anching is wholly passive: 'wahrscheinlicher ist es wohl, daß hier ein im rachealsystem autonom auftretender Gliederungsprozeß zum mindesten itbeteiligt ist'.

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CONTENTS

Montagna, W., Eisen, A. Z., & Goldman, A. S. The Tinctorial Behaviour of Human Mast Cells

Rabinovitch, M., Mota, I., & Yoneda, S. Note on the Histochemical Localization of Glycogen and Pentosepolynucleotides in the Visual Cells of the Chick (*Gallus gallus*)

Dilworth, S., & Appleyard, H. M. A Sharpening Machine for a New Type of Microtome Knife

Millott, N., & Lynn, W. G. The Effect of Phenylthiourea on Pigmentation by Melanin in the Developing Frog, *Eleutherodactylus martinicensis*

Brewer, D. B. Histological and Polarization Studies of the Brush Border of the Proximal Convoluted Tubules of the Rat Kidney

Jones, W. C. The Orientation of the Optic Axis of Spicules of *Leucosolenia complicata*

Wolfe, L. S. The Deposition of the Third Instar Larval Cuticle of *Calliphora erythrocephala*

Wolfe, L. S. Studies of the Development of the Imaginal Cuticle of *Calliphora erythrocephala*

Carlisle, D. B. On the Relationship between Mammary, Sweat, and Sebaceous Glands

Horridge, A. Observations on the Nerve Fibres of *Aurellia aurita*

Shafiq, S. A. A Study of the Embryonic Development of the Gooseberry Sawfly, *Pteronidea ribesii*

Wigglesworth, V. B. Growth and Regeneration in the Tracheal System of an Insect, *Rhodnius prolixus* (Hemiptera)

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